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(54) Title: **MODIFIED CARBOCYANINE DYES AND THEIR CONJUGATES**

(57) Abstract: Chemically reactive carbocyanine dyes incorporating an indolium ring moiety that is substituted at the 3-position by a reactive group or by a conjugated substance, and their uses, are described. Conjugation through this position results in spectral properties that are uniformly superior to those of conjugates of spectrally similar dyes wherein attachment is at a different position. The invention includes derivative compounds having one or more benzo nitrogens.

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MODIFIED CARBOCYANINE DYES AND THEIR CONJUGATES**FIELD OF THE INVENTION**

5 The invention relates to colored and fluorescent chemicals, including reactive dyes and dye-conjugates; and to their uses.

BACKGROUND OF THE INVENTION

10 Fluorescent compounds are covalently or noncovalently attached to other materials to impart color and fluorescence. Brightly fluorescent dyes permit detection or location of the attached materials with great sensitivity. Certain carbocyanine dyes have demonstrated utility as labeling reagents for a variety of biological applications, e.g. U.S. Patents 4,981,977 to Southwick, et al. (1991);
15 5,268,486 to Waggoner et al. (1993); 5,569,587 to Waggoner (1996); 5,569,766 to Waggoner et al. (1996); 5,486,616 to Waggoner et al. (1996); 5,627,027 to Waggoner (1997); 5,808,044 to Brush, et al. (1998); 5,877,310 to Reddington, et al. (1999); 6,002,003 to Shen, et al. (1999); 6,004,536 to Leung et al. (1999); 6,008,373 to Waggoner, et al. (1999); 6,043,025 to Minden, et al. (2000); 6,127,134 to Minden, et al.
20 (2000); 6,130,094 to Waggoner, et al. (2000); 6,133,445 to Waggoner, et al. (2000); also WO 97/40104, WO 99/51702, WO 01/21624, and EP 1 065 250 A1; and TETRAHEDRON LETTERS 41, 9185-88 (2000); all of the above incorporated by reference. Nevertheless, many carbocyanine dyes are known to share certain disadvantages, e.g. severe quenching of the fluorescence of carbocyanine dyes in
25 biopolymer conjugates, e.g. quenching of Cy5 and Cy7 dye variants on conjugates, as discussed by Gruber et al., BIOCONJUGATE CHEM. 11, 696 (2000), and in EP 1 065 250 A1, 0004. In addition, certain desired sulfoalkyl derivatives of the reactive carbocyanine dyes are difficult to prepare, as indicated for Cy3 and Cy5 variants by Waggoner and colleagues in BIOCONJUGATE CHEM. 4, 105, 109 col.2 (1993).
30 Cyanine dyes also have a very strong tendency to self-associate (i.e. stack), which can significantly reduce the fluorescence quantum yields, as described in the extensive review by Mishra, et al., CHEM. REV. 100, 1973 (2000).

Modification of an indolium ring of the carbocyanine dye to permit a reactive

group or conjugated substance at the number 3 position unexpectedly mitigates these problems and results in dye-conjugates that are uniformly and substantially more fluorescent on proteins, nucleic acids and other biopolymers, than conjugates labeled with structurally similar carbocyanine dyes bound through the nitrogen atom at the number 1 position. In addition to having more intense fluorescence emission than structurally similar dyes at virtually identical wavelengths, and decreased artifacts in their absorption spectra upon conjugation to biopolymers, certain embodiments of the invention also have greater photostability and higher absorbance (extinction coefficients) at the wavelength(s) of peak absorbance than such structurally similar dyes. These improvements result in significantly greater sensitivity in assays that use these dyes and their conjugates, while utilizing available filters and instrumentation already commercially available for use with similar dyes.

Furthermore, the dyes of the invention typically exhibit absorbance maxima between about 530 nm and about 800 nm, so dyes can be selected to match the principal emission lines of the mercury arc lamp (546 nm), frequency-doubled Nd-Yag laser (532 nm), Kr-ion laser (568 nm and 647 nm), HeNe laser (543 nm, 594 nm, and 633 nm) or long-wavelength laser diodes (especially 635 nm and longer). The azacarbocyanine dyes of the invention exhibit a bathochromic spectral shift (a shift to longer wavelength) of approximately 20 to 50 nm relative to otherwise structurally similar carbocyanine dyes known in the art. Some dyes of the invention exhibit very long wavelength excitation (at least 640 nm, but some greater than about 730 nm) and emission bands (at least 665 nm, and some greater than about 750 nm), so they are particularly useful for samples that are transparent to infrared wavelengths.

DESCRIPTION OF DRAWINGS

Figure 1. Direct comparison of absorbance properties of Compound 30 (Cy5-type linkage, open circles) with Compound 24 (of the invention, closed circles) when conjugated to GAR at DOS's of approximately 2.8, 4.3, and 5.5 (600 nm absorbance bands increasing as a function of increasing DOS, 650 nm absorbance bands normalized to 1.0) (see Example 47).

Figure 2. Comparison of the fluorescence of goat anti-rabbit IgG (GAR) conjugates of Compound 13 (solid line) and those of the spectrally similar CY3 dye (dashed line) (see Example 49).

- 5 Figure 3. Photostability comparison of Compound 9 (solid circles) with Cy5 (open circles), (40X objective, pH = 7.5 solution) (see Example 50).

Figure 4. Comparison of the change in absorbance of Compound 9 (closed circles) and Cy5 (open circles) upon DNA incorporation using ULS methodology (see Example 58).

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Figure 5. Comparison of the absorption and fluorescence properties of identical concentrations of Compound 9 (solid line) and Cy5 (dashed line) labeled cDNA (see Example 60).

15 **SUMMARY OF THE INVENTION AND DESCRIPTION OF PREFERRED EMBODIMENTS**

The present invention describes modified carbocyanine dyes and their conjugates. Preferred compounds have at least one substituted indolium ring system wherein the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. Other preferred compounds incorporate an azabenzazolum ring moiety and at least one sulfonate moiety. The dyes and dye conjugates are used to locate or detect the interaction or presence of analytes or ligands in a sample. Kits incorporating such dyes or dye conjugates facilitate their use in such methods.

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Dyes

The carbocyanine dyes of the invention typically comprise two heterocyclic ring systems bound together by a polymethine linker, according to the formula:

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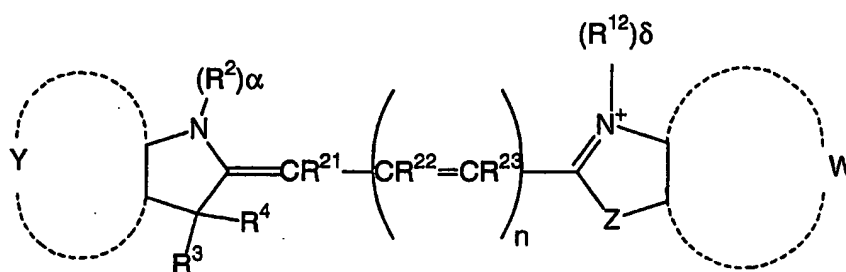


where A is a first heterocyclic ring system that is a substituted benzazolum ring that optionally incorporates one or more nitrogen atoms (azabenzazolum rings), B is a

second heterocyclic ring system that is a substituted benzazolum or azabenzazolum ring, and BRIDGE is a polymethine linker that is optionally substituted. The first and second ring systems and polymethine linker are optionally further substituted by a variety of substituents or are fused to additional rings that are optionally further substituted. In all aspects of the invention, the carbocyanine dye contains a chemically reactive group or a conjugated substance that is attached at carbon 3 of an indolium ring system. In a preferred embodiment, the carbocyanine dye is further substituted one or more times by sulfo or sulfoalkyl.

By "sulfo" is meant sulfonic acid, or salt of sulfonic acid (sulfonate). Similarly, by "carboxy" is meant carboxylic acid or salt of carboxylic acid. "Phosphate", as used herein, is an ester of phosphoric acid, and includes salts of phosphate. "Phosphonate", as used herein, means phosphonic acid and includes salts of phosphonate. As used herein, unless otherwise specified, the alkyl portions of substituents such as alkyl, alkoxy, arylalkyl, alylamino, dialkylamino, trialkylammonium, or perfluoroalkyl are optionally saturated, unsaturated, linear or branched, and all alkyl, alkoxy, alkylamino, and dialkylamino substituents are optionally themselves further substituted by carboxy, sulfo, amino, or hydroxy.

A preferred embodiment is a compound of the formula:



Formula X

and its salts, wherein Y represents the atoms necessary to form one to two fused aromatic rings having 6 atoms in each ring, which atoms are selected from -CH, -C, -CR¹, and -N(R²)_β, where each β is 0 or 1, and each R¹ is independently -L-R_x; or -L-S_c; or amino, sulfo, trifluoromethyl, or halogen; or C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino, optionally further substituted. Incorporation of one

or more non-hydrogen substituents on the fused rings can be used to fine tune the absorption and emission spectrum of the resulting dye. In one embodiment, there is at least one non-hydrogen substituent, preferably sulfo, an alkoxy or halogen; preferably the halogen is bromine.

5

The substituents R^3 and R^4 , which may be the same or different, are alkyl or arylalkyl, and optionally further substituted. Preferably, R^3 is $-L-R_x$ or $-L-S_x$ (as defined below). The substituents R^2 , R^4 , R^{12} , R^{13} and R^{14} , are independently $-L-R_x$; or $-L-S_x$; or a C_1-C_{22} alkyl or C_7-C_{22} arylalkyl, each alkyl portion of which optionally incorporates up to six hetero atoms, selected from N, O and S, and each alkyl portion of which is optionally substituted one or more times by fluorine, chlorine, bromine, iodine, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, C_1-C_6 alkoxy, C_1-C_6 alkylamino, or C_2-C_{12} dialkylamino, or C_3-C_{18} trialkylammonium. Preferably, R^4 , R^{13} and R^{14} are independently alkyl having 1-6 carbons, optionally substituted one or more times by fluorine, chlorine, bromine, iodine, hydroxy, carboxy, sulfo, or amino; more preferably R^4 , R^{13} and R^{14} are methyl or ethyl. In one aspect of the invention, R^4 , R^{13} and R^{14} are methyl. Alternatively, R^4 taken in combination with R^{21} forms a 6-membered ring that is optionally substituted by alkyl having 1-6 carbons, or R^4 taken in combination with R^3 forms a saturated or unsaturated ring, that is substituted by $-L-R_x$ or $-L-S_x$. Likewise, R^{13} and R^{14} , taken in combination, optionally complete a five- or six-membered saturated or unsaturated ring, that is optionally substituted by $-L-R_x$; or $-L-S_x$, or R^{14} taken in combination with one of R^{23} forms a 6-membered ring that is optionally substituted by alkyl having 1-6 carbons.

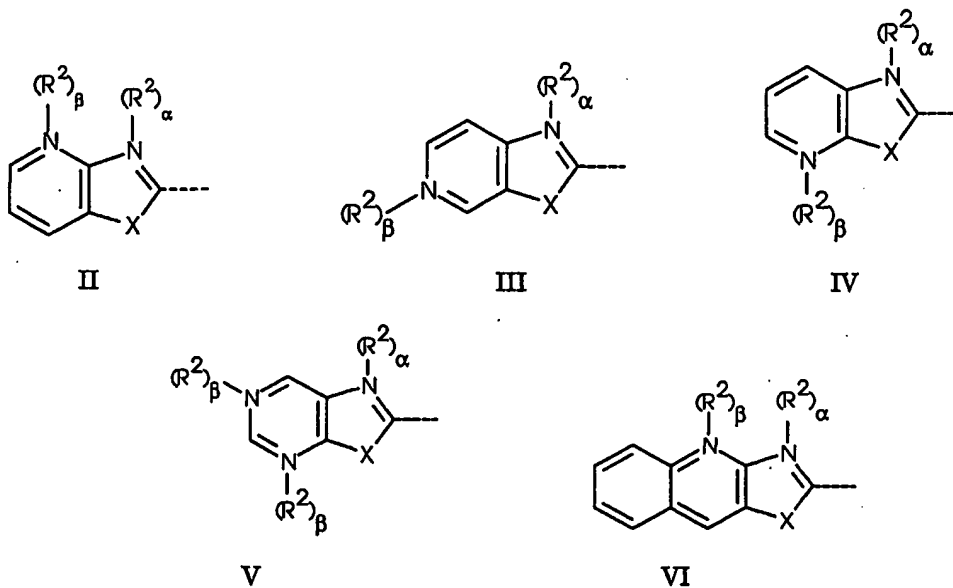
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Preferably R^2 and R^{12} are independently alkyl with 1-6 carbon atoms that are unsubstituted or are substituted once by hydroxy, sulfo, carboxy or amino. Where either R^2 or R^{12} is substituted by hydroxy, sulfo, carboxy or amino, the substituent is preferably separated from the indolium or other benzazolium nitrogen atom by 2-6 carbon atoms. Where R^2 and R^{12} are unsubstituted alkyl groups, they are preferably methyl or ethyl, most preferably methyl. Typically R^2 and R^{12} are the same and are methyl, ethyl, sulfopropyl or sulfobutyl.

30

Similarly, W represents the atoms necessary to form one to two fused aromatic rings having 6 atoms in each ring, which atoms are selected from -CH, -C, -CR¹, and -N(R¹²)_β, where each β' is 0 or 1, and each R¹ is independently -L-R_α; or -L-S_α; or amino, sulfo, trifluoromethyl, or halogen; or C₁-C₈ alkyl, C₁-C₈ alkoxy, C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy. Where the six membered rings form an azabenzazole ring system, they typically incorporate 1-3 nitrogen atoms, more preferably 1-2 nitrogen atoms, typically incorporated in the first 6-membered aromatic ring fused to the azole ring. In one aspect of the invention, the ring system W contains only carbon atoms and is a benzazole ring system.

Where A or B is an azabenzazolium, its fused aromatic ring(s) typically incorporate 1-3 nitrogen atoms, more preferably 1-2 nitrogen atoms, typically incorporated in the first 6-membered aromatic ring fused to the azole ring. Preferred embodiments of the azabenzazole moiety include without limitation the following structures, (and the equivalent structures where the nitrogen is quaternized by R¹²), where X corresponds to -CR³R⁴:



When Y or W includes one or more nitrogen atoms, at least one of the azabenzazole nitrogen atoms is quaternized, resulting in a formal positive charge. In

one embodiment, the azole nitrogen atom is quaternized, and the benzo nitrogen atom is unsubstituted. Preferably, the azole nitrogen atom is unsubstituted and at least one benzo nitrogen atom is quaternized. Typically, no more than one azole nitrogen on a given azabenzazole is quaternized, i.e. α is 0 or 1, β is 0 or 1, and $\alpha + \text{all } \beta = 1$; and δ is 0 or 1, β' is 0 or 1, and $\delta + \text{all } \beta' = 1$. The nitrogen atom shifts the emission spectra to a longer wavelength, relative to dyes having a carbon atom at the same position. The presence of additional fused 6-membered rings (as in Formula VI, above) shift the wavelength even further.

Choice of the X and Z moieties may also affect the dye's absorption and fluorescence emission properties. X and Z are optionally the same or different, and spectral properties of the resulting dye may be tuned by careful selection of X and Z. In one embodiment, Z is one of O, S, Se or NR^{15} , where R^{15} is H or an alkyl group having 1-22 carbons, that is optionally substituted one or more times by hydroxy, carboxy, sulfo, amino, alkylamino having 1-6 carbons or dialkylamino having 2-12 carbons. Alternatively, Z is O, S, or $-\text{CR}^{13}\text{R}^{14}$, where R^{13} and R^{14} , which may be the same or different, are alkyl or arylalkyl, and optionally further substituted. Typically X and Z are $-\text{CR}^3\text{R}^4$ and $-\text{CR}^{13}\text{R}^{14}$, respectively.

Where Z is $-\text{CR}^{13}\text{R}^{14}$, the substituents R^{13} and R^{14} , which may be same or different, are independently $-\text{L-R}_x$; or $-\text{L-S}_y$; or a $\text{C}_1\text{-C}_{22}$ alkyl or $\text{C}_7\text{-C}_{22}$ arylalkyl, each alkyl portion of which optionally incorporates up to six hetero atoms, selected from N, O and S, and each alkyl portion of which is optionally substituted one or more times by fluorine, chlorine, bromine, iodine, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, $\text{C}_1\text{-C}_6$ alkoxy, $\text{C}_1\text{-C}_6$ alkylamino, or $\text{C}_2\text{-C}_{12}$ dialkylamino, or $\text{C}_3\text{-C}_{18}$ trialkylammonium. Alternatively R^{13} and R^{14} in combination complete a five or six membered saturated or unsaturated ring that is optionally substituted by $-\text{L-R}_x$; or $-\text{L-S}_y$, or combine with a methine substituent to form a ring, as described below. Preferably R^{13} and R^{14} are independently alkyl with 1-6 carbon atoms that are unsubstituted or are substituted once by hydroxy, sulfo, carboxy or amino. Where either R^{13} or R^{14} is substituted by hydroxy, sulfo, carboxy or amino, the substituent is preferably separated from the indolium or other benzazolium nitrogen atom by 2-6 carbon atoms. In one aspect of the invention, R^{13} and R^{14} are alkyls having 1-6 carbons, preferably methyls. In another aspect of the invention, one of R^{13}

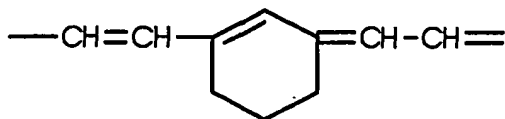
and R^{14} is methyl, and the other is alkyl having 1-6 carbons that is substituted by carboxy or by sulfo or by hydroxy, or by $-L-R_x$ or $-L-Sc$.

The length of the polymethine BRIDGE between the heterocyclic ring systems affects the dye's absorption and emission properties. Typically, $n=1, 2$, or 3 . Where n is >3 , the dyes have spectra even further shifted into the infrared region. In a preferred aspect of the azacarbocyanine dyes, n is 2 . Where Z is $CR^{18}R^{14}$, and a and $b = 0$, and the indolium heterocycle is not fused to additional rings, the resulting "indocarbocyanine" dye typically exhibits an absorption maximum near 550 nm.

Where $a = 1$ and $b = 0$, the "indodicarbocyanines" typically absorb maximally near 650 nm and are termed. The "indotricarbocyanine" dyes, where a and b are both 1 , typically absorb maximally near 750 nm.

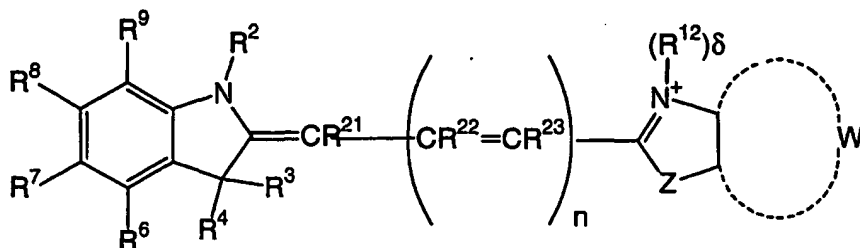
Each of R^{21} , R^{22} , and R^{23} , when present, is independently H, F, Cl, alkyl having 1-6 carbons, alkoxy having 1-6 carbons, aryloxy, a N-heteroaromatic moiety, or an iminium ion. Alternatively, two adjacent substituents, when taken in combination, form a 4-, 5-, or 6-membered saturated or unsaturated hydrocarbon ring that is unsubstituted or is optionally substituted one or more times by a saturated or unsaturated alkyl having 1-6 carbons, halogen, or a carbonyl oxygen. In yet another embodiment, R^{21} combines with R^4 to form a 6-membered ring that is optionally substituted by alkyl having 1-6 carbons. Alternatively, the methine substituent adjacent to Z , taken in combination with one of R^{13} and R^{14} , forms a 6-membered ring that is optionally substituted by alkyl having 1-6 carbons.

Typically, each of R^{21} , R^{22} , and R^{23} is H. Where one of R^{21} , R^{22} , and R^{23} is nonhydrogen, it is typically the substituent on the center carbon of BRIDGE. Similarly, where BRIDGE incorporates a 4-, 5-, or 6-membered ring, it typically occurs at the center of the BRIDGE moiety, for instance as shown below for a pentamethine dye:



Formula IX

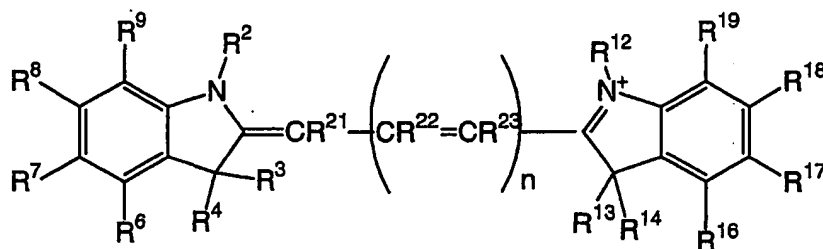
A preferred version of the invention has the formula:



Formula XI

The substituents R^6 - R^9 are independently selected from H, alkyl having from 1-6 carbons, alkoxy having 1-6 carbons, amino, alkylamino having 1-6 carbons, or dialkylamino having 2-12 carbons, sulfo, carboxy, perfluoroalkyl having 1-6 carbons, or halogen.

In one aspect of the invention, both A and B are benzazolum rings, according to the formula:



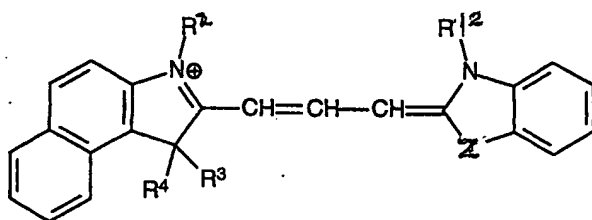
Formula XXI

where the substituents R^{16} - R^{19} are independently selected from H, alkyl having from 1-6 carbons, alkoxy having 1-6 carbons, amino, alkylamino having 1-6 carbons, or dialkylamino having 2-12 carbons, sulfo, carboxy, perfluoroalkyl having 1-6 carbons, or halogen.

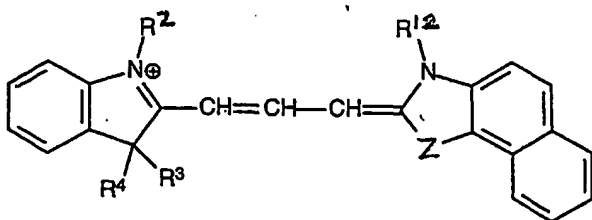
Incorporation of one or more non-hydrogen substituents on either or both

benzazolum rings are useful to fine-tune the absorption and emission spectrum. There is typically at least one non-hydrogen substituent on each of the benzazolum rings, preferably sulfo, an alkoxy or a halogen substituent. For the benzazolum dyes, the substituents on the benzo rings are typically H or sulfo. In one embodiment, one of R⁶, R⁷, R⁸, and R⁹ or of R¹⁶, R¹⁷, R¹⁸, and R¹⁹ is a dialkylamino that is a saturated 5- or 6-membered nitrogen heterocycle, such as piperidine. Additionally, any two adjacent substituents of R⁶-R⁹ and R¹⁶-R¹⁹ are optionally taken in combination to form one or more fused aromatic rings. These additional rings are optionally further substituted as described above for R⁶-R⁹ and R¹⁶-R¹⁹, and in particular by sulfonic acids.

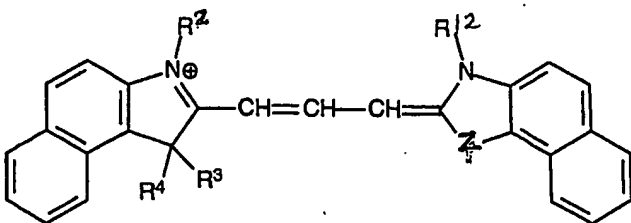
Selected examples of carbocyanine dyes of the invention possessing additional fused aromatic rings are given below (for simplicity, all but a few of the possible substituents are shown as hydrogen, with the shortest polymethine bridge):



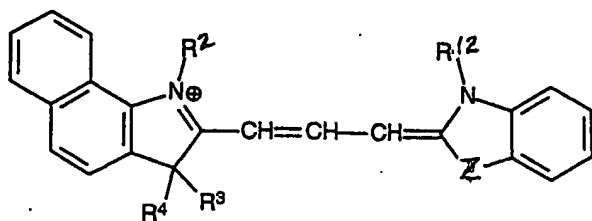
Formula XII



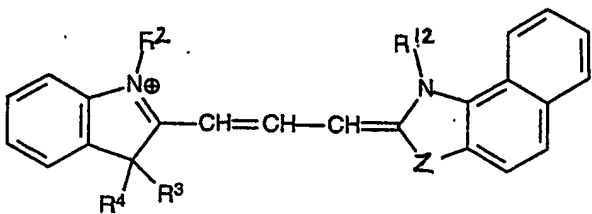
Formula XIII



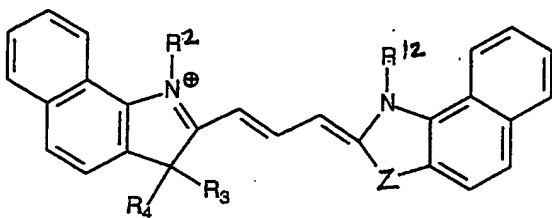
Formula XIV



Formula XV

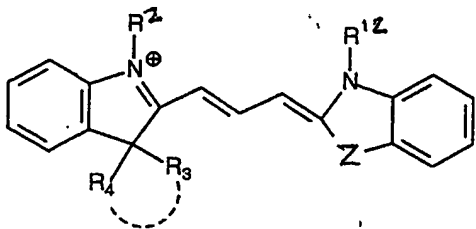


Formula XVI

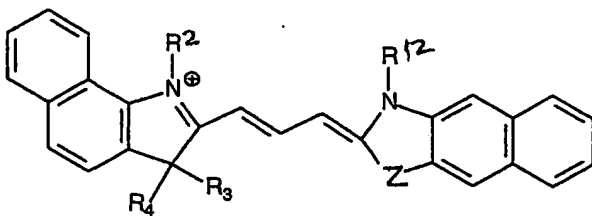


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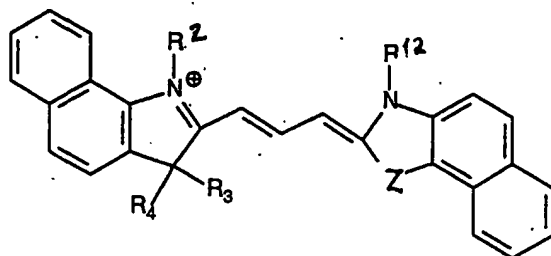
Formula XVII



Formula XVIII



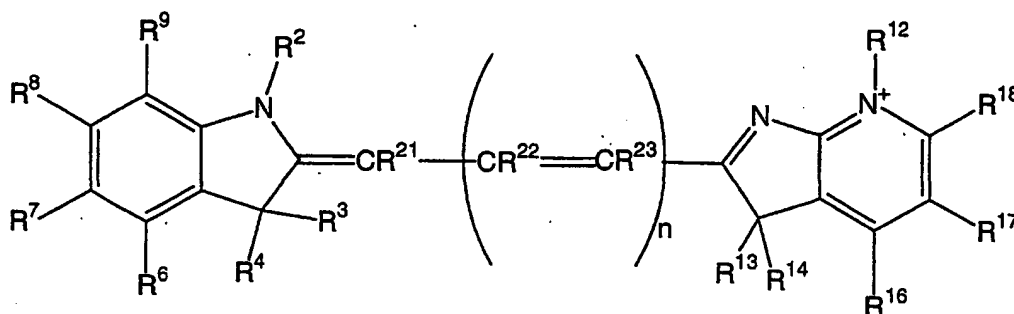
Formula XIX



Formula XX

These basic structures, and their longer-wavelength analogs, are optionally further substituted as described in this section. Additional variants not specifically depicted above are also within the scope of the invention, e.g the "left-hand" indolium of Formula XVIII linked to a benzazolinium of Formula XVII, XIX, or XX.

In another aspect of the invention, A is a benzazolinium and B is an azabenzazolinium, according to the formula:



Formula XXII

where the substituents R^6 - R^9 and R^{16} - R^{19} are as described previously. Preferred substituents for R^{16} through R^{18} are independently H, $-L-R_x$; or $-L-S_x$; or amino, trifluoromethyl, or halogen; or C_1 - C_8 alkyl, C_1 - C_8 alkoxy, C_1 - C_8 alkylamino, C_2 - C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy. Preferably, the only non-hydrogen substituent on the B side, when present, is a bromine at preferably bromine at R^{17} . Preferably $n=2$.

In one aspect of the invention, the carbocyanine dyes of the invention are sulfonated one or more times. If the dye of the invention is substituted by sulfo, it is

typically sulfonated at R⁷ or R¹⁷ or both, or sulfoalkylated at R² or R¹² or both, or is both sulfonated and sulfoalkylated. Typically, where the aromatic ring of Y or W contains one or more nitrogen atoms, the ring is not sulfonated. Generally, commercially available reactive carbocyanine dyes are sulfonated up to three times (at positions corresponding to R⁷ and R¹⁷, and as sulfoalkyl at one of R² and R¹²), leaving one of R² and R¹² for the location of the reactive group. In contrast, by attaching the reactive group (or conjugated substance) at R³, certain carbocyanine dyes of the invention may be sulfonated at least four times (at R⁷, at R¹⁷, and as sulfoalkyl at R² and R¹²). This extra sulfonation, as well as the change in attachment site, results in reactive dyes and dye conjugates that are brighter, more soluble in aqueous solutions, and more resistant to the fluorescence quenching that results from dye-dye stacking interactions. However, sulfonation by four or more sulfonic acids is not required for the dyes of the invention to have spectral properties that are superior to those of structurally similar dyes that are not linked through the 3 position of the indolium ring (Figure 4).

In addition, the dyes of the invention are substituted by one or more chemically reactive groups (-L-R_x) or conjugated substances (-L-Sc), as described below. Typically, the -L-R_x or -L-Sc moieties are bound to the dye at an R², R³, R⁴, R¹², R¹³ or R¹⁴. Alternatively, -L-R_x or -L-Sc is bound to the dye at an aromatic carbon atom of the azabenzazolum ring, or the benzazolum ring. In one embodiment, one or more of R² and R¹² is -L-R_x or -L-Sc. In yet another preferred embodiment of the invention, one or more of R³, R⁴, R¹³, and R¹⁴ is -L-R_x or -L-Sc. Alternatively, one or more of R²¹, R²², and R²³ is -L-R_x or -L-Sc. In a preferred embodiment, the dye of the invention is substituted by only one -L-R_x or -L-Sc.

Many embodiments of the compounds of the invention possess an overall electronic charge. It is to be understood that when such electronic charges are shown to be present, they are balanced by the presence of an appropriate counterion, which may or may not be explicitly identified. A biologically compatible counterion, which is preferred for some applications, is not toxic in biological applications, and does not have a substantially deleterious effect on biomolecules. Where the compound of the invention is positively charged, the counterion is typically selected from, but not limited to, chloride, bromide, iodide, sulfate, alkanesulfonate, arylsulfonate,

phosphate, perchlorate, tetrafluoroborate, tetraarylboride, nitrate and anions of aromatic or aliphatic carboxylic acids. Where the compound of the invention is negatively charged, the counterion is typically selected from, but not limited to, alkali metal ions, alkaline earth metal ions, transition metal ions, ammonium or substituted ammonium or pyridinium ions. Preferably, any necessary counterion is biologically compatible, is not toxic as used, and does not have a substantially deleterious effect on biomolecules. Counterions are readily changed by methods well known in the art, such as ion-exchange chromatography, or selective precipitation.

It is to be understood that the dyes of the invention have been drawn in one or another particular electronic resonance structure. Every aspect of the instant invention applies equally to dyes that are formally drawn with other permitted resonance structures, as the electronic charge on the subject dyes are delocalized throughout the dye itself.

Table 1. Spectral properties of selected dyes of the invention

Cpd No.	Excitation (nm, MeOH)	Emission (nm, MeOH)	Quantum Yield† (MeOH)
63	665	694	0.55
64	653	695	0.15
65	664	693	0.4
66	686	706	0.35
68	664	696	0.4
70	570	592	0.44
72	663	694	0.42
73	664	697	0.48
76	685	705	0.46
81	750	800	0.12

† Relative to Nile blue in ethanol and CY5 dye in methanol

Conjugates of Reactive Dyes

In one embodiment of the invention, the dye contains at least one group -L-R_x,

where R_x is the reactive group that is attached to the dye by a covalent linkage L. In certain embodiments, the covalent linkage attaching the dye to R_x contains multiple intervening atoms that serve as a spacer. The dyes with a reactive group (R_x) label a wide variety of organic or inorganic substances that contain or are modified to

5 contain functional groups with suitable reactivity; resulting in chemical attachment of the conjugated substance (S_c), represented by $-L-S_c$. As used herein, "reactive group" means moiety on the compound that is capable of chemically reacting with a functional group on a different compound to form a covalent linkage. Typically the reactive group is an electrophile or nucleophile that can form a covalent linkage

10 through exposure to the corresponding functional group that is a nucleophile or electrophile, respectively. Alternatively, the reactive group is a photoactivatable group, and becomes chemically reactive only after illumination with light of an appropriate wavelength. Typically, the conjugation reaction between the reactive dye and the substance to be conjugated results in one or more atoms of the reactive group

15 R_x to be incorporated into a new linkage L attaching the dye to the conjugated substance S_c . Selected examples of reactive groups and linkages are shown in Table 2, where the reaction of an electrophilic group and a nucleophilic group yields a covalent linkage.

20 Table 2: Examples of some routes to useful covalent linkages

Electrophilic Group	Nucleophilic Group	Resulting Covalent Linkage
activated esters*	amines/anilines	carboxamides
acrylamides	thiols	thioethers
acyl azides**	amines/anilines	carboxamides
acyl halides	amines/anilines	carboxamides
acyl halides	alcohols/phenols	esters
acyl nitriles	alcohols/phenols	esters
acyl nitriles	amines/anilines	carboxamides
aldehydes	amines/anilines	imines
aldehydes or ketones	hydrazines	hydrazones
aldehydes or ketones	hydroxylamines	oximes

alkyl halides	amines/anilines	alkyl amines
alkyl halides	carboxylic acids	esters
alkyl halides	thiols	thioethers
alkyl halides	alcohols/phenols	ethers
alkyl sulfonates	thiols	thioethers
alkyl sulfonates	carboxylic acids	esters
alkyl sulfonates	alcohols/phenols	ethers
anhydrides	alcohols/phenols	esters
anhydrides	amines/anilines	carboxamides
aryl halides	thiols	thiophenols
aryl halides	amines	aryl amines
aziridines	thiols	thioethers
boronates	glycols	boronate esters
carbodiimides	carboxylic acids	N-acylureas or anhydrides
dialkyl azoalkanes	carboxylic acids	esters
epoxides	thiols	thioethers
haloacetamides	thiols	thioethers
haloplatinate	amino	platinum complex
haloplatinate	heterocycle	platinum complex
haloplatinate	thiol	platinum complex
halotriazines	amines/anilines	aminotriazines
halotriazines	alcohols/phenols	triazinyl ethers
imido esters	amines/anilines	amidines
isocyanates	amines/anilines	ureas
isocyanates	alcohols/phenols	urethanes
isothiocyanates	amines/anilines	thioureas
maleimides	thiols	thioethers
phosphoramidites	alcohols	phosphite esters
silyl halides	alcohols	silyl ethers
sulfonate esters	amines/anilines	alkyl amines
sulfonate esters	thiols	thioethers
sulfonate esters	carboxylic acids	esters

sulfonate esters	alcohols	ethers
sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

* Activated esters, as understood in the art, generally have the formula $-\text{CO}\Omega$, where Ω is a good leaving group (e.g. succinimidyl (- $\text{OC}_4\text{H}_4\text{O}_2$) sulfosuccinimidyl (- $\text{OC}_4\text{H}_3\text{O}_2\text{-SO}_3\text{H}$), -1-oxybenzotriazolyl (- $\text{OC}_6\text{H}_4\text{N}_3$); or an aryloxy group or aryloxy substituted one or more times by electron withdrawing substituents such as nitro, fluoro, chloro, cyano, or trifluoromethyl, or combinations thereof, used to form activated aryl esters; or a carboxylic acid activated by a carbodiimide to form an anhydride or mixed anhydride $-\text{OCOR}^a$ or $-\text{OCNR}^a\text{NHR}^b$, where R^a and R^b , which may be the same or different, are $\text{C}_1\text{-C}_6$ alkyl, $\text{C}_1\text{-C}_6$ perfluoroalkyl, or $\text{C}_1\text{-C}_6$ alkoxy; or cyclohexyl, 3-dimethylaminopropyl, or N-morpholinoethyl).

** Acyl azides can also rearrange to isocyanates

The covalent linkage L binds the reactive group R_x or conjugated substance S_o to the compound, either directly (L is a single bond) or with a combination of stable chemical bonds, optionally including single, double, triple or aromatic carbon-carbon bonds, as well as carbon-nitrogen bonds, nitrogen-nitrogen bonds, carbon-oxygen bonds, carbon-sulfur bonds, phosphorus-oxygen bonds, phosphorus-nitrogen bonds, and nitrogen-platinum bonds. L typically includes ether, thioether, carboxamide, sulfonamide, urea, urethane or hydrazine moieties. In one embodiment, the covalent linkage incorporates a platinum atom, such as described in U.S. Patent No. 5,714,327 (incorporated by reference). Preferred L moieties have 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S; and are composed of any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. Preferably L is a combination of single carbon-carbon bonds and carboxamide or thioether bonds. The longest linear segment of the linkage L preferably contains 4-10 nonhydrogen atoms, including one or two heteroatoms. Examples of L include substituted or unsubstituted polymethylene, arylene, alkylarylene, arylenealkyl, or arylthio. In one embodiment, L contains 1-6 carbon atoms; in another, L comprises a thioether linkage. In yet another embodiment, L is or incorporates the formula $-(\text{CH}_2)_d(\text{CONH}(\text{CH}_2)_e)_{z'}$, or $-(\text{CH}_2)_d(\text{CON}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_e)_{z'}$, $-(\text{CH}_2)_d(\text{CONH}(\text{CH}_2)_e\text{NH}_2)_{z'}$, $-(\text{CH}_2)_d(\text{CONH}(\text{CH}_2)_e\text{NHCO})_{z'}$, where d is 0-5, e is 1-5 and z' is 0 or 1.

Choice of the reactive group used to attach the dye to the substance to be

conjugated typically depends on the functional group on the substance to be conjugated and the type or length of covalent linkage desired. The types of functional groups typically present on the organic or inorganic substances include, but are not limited to, amines, amides, thiols, alcohols, phenols, aldehydes, ketones, phosphates, imidazoles, hydrazines, hydroxylamines, disubstituted amines, halides, epoxides, carboxylate esters, sulfonate esters, purines, pyrimidines, carboxylic acids, olefinic bonds, or a combination of these groups. A single type of reactive site may be available on the substance (typical for polysaccharides), or a variety of sites may occur (e.g. amines, thiols, alcohols, phenols), as is typical for proteins. A conjugated substance may be conjugated to more than one dye, which may be the same or different, or to a substance that is additionally modified by a hapten, such as biotin. Although some selectivity can be obtained by careful control of the reaction conditions, selectivity of labeling is best obtained by selection of an appropriate reactive dye.

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Typically, R_X will react with an amine, a thiol, an alcohol, an aldehyde or a ketone. Preferably R_X reacts with an amine or a thiol functional group. In one embodiment, R_X is an acrylamide, a reactive amine (including a cadaverine or ethylenediamine), an activated ester of a carboxylic acid (typically a succinimidyl ester of a carboxylic acid), an acyl azide, an acyl nitrile, an aldehyde, an alkyl halide, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a carboxylic acid, a diazoalkane, a haloacetamide, a halotriazine, a hydrazine (including hydrazides), an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a sulfonyl halide, or a thiol group. By "reactive platinum complex" is particularly meant chemically reactive platinum complexes such as described in U.S. Patent Nos. 5,580,990; 5,714,327; 5,985,566 (all incorporated by reference).

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Where the reactive group is a photoactivatable group, such as an azide, diazirinyl, azidoaryl, or psoralen derivative, the dye becomes chemically reactive only after illumination with light of an appropriate wavelength.

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Where R_X is an activated ester of a carboxylic acid, the reactive dye is particularly useful for preparing dye-conjugates of proteins, nucleotides,

oligonucleotides, or haptens. Where R_x is a maleimide or haloacetamide the reactive dye is particularly useful for conjugation to thiol-containing substances. Where R_x is a hydrazide, the reactive dye is particularly useful for conjugation to periodate-oxidized carbohydrates and glycoproteins, and in addition is an aldehyde-fixable polar tracer for cell microinjection.

Preferably, R_x is a carboxylic acid, a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide group, an aliphatic amine, a perfluorobenzamido, an azidoperfluorobenzamido group, or a psoralen. More preferably, R_x is a succinimidyl ester of a carboxylic acid, a maleimide, an iodoacetamide, or a reactive platinum complex. In a particular embodiment R_x is a reactive platinum complex, or a succinimidyl ester of a carboxylic acid. Where R_x is a reactive platinum complex, it is typically a haloplatinate or a platinum nitrate.

Based on the above-mentioned attributes, the appropriate reactive dye of the invention is selected for the preparation of the desired dye-conjugate, whose advantageous properties make them useful for a wide variety of applications. When compared to conjugates of widely used carbocyanine dyes for which the point of attachment is at the 1-position of the indolium moiety (e.g. Amersham's Cy dyesTM), the dye-conjugates of this invention have demonstrably superior optical properties. See, e.g. Example 49 comparing protein conjugates of Cy3 dye and spectrally similar conjugates of compound 13 of the invention (wherein $n = 1$); Tables 2 and 4 with an extensive comparison of protein conjugates of Cy5 dye with the spectrally similar conjugates of compound 9 of the invention (wherein $n=2$); Examples 45-46, 48, and 50; and Figures 2-3. See also, Example 52 comparing dye-fluorophore conjugates; as well as Table 5, Examples 58-60 comparing dye-nucleic acid conjugates; and Examples 48 and 52, and for flow-cytometric comparison of cell populations labeled with dye-conjugates. The desired dye-conjugate is selected based on the intended application.

Particularly useful dye-conjugates include, among others, conjugates where Sc is an antigen, steroid, vitamin, drug, hapten, metabolite, toxin, environmental pollutant, amino acid, peptide, protein, nucleic acid, nucleic acid polymer, carbohydrate, lipid, ion-complexing moiety, or glass, plastic or other non-biological

polymer. Alternatively, Sc is a cell, cellular system, cellular fragment, or subcellular particle, e.g. inter alia, a virus particle, bacterial particle, virus component, biological cell (such as animal cell, plant cell, bacteria, yeast, or protist), or cellular component. Reactive dyes typically label functional groups at the cell surface, in cell membranes, organelles, or cytoplasm.

Typically Sc is an amino acid, peptide, protein, tyramine, polysaccharide, ion-complexing moiety, nucleoside, nucleotide, oligonucleotide, nucleic acid, hapten, psoralen, drug, hormone, lipid, lipid assembly, polymer, polymeric microparticle, biological cell or virus. More typically, Sc is a peptide, a protein, a nucleotide, an oligonucleotide, or a nucleic acid. When conjugating dyes of the invention to such biopolymers, it is possible to incorporate more dyes per molecule to increase the fluorescent signal. For example, it is possible to incorporate at least four molecules of such dyes per molecule of antibody without loss of total fluorescence, whereas fluorescence of the spectrally comparable Cy5 (wherein $n = 2$) is strongly quenched when greater than approximately two Cy5 dyes are incorporated per antibody. These results confirm problems with Cy dye conjugates reported by others, e.g. BIOCONJUGATE CHEM. 11, 696 (2000). A comparison of commercially available Cy5 conjugates or conjugates of known dyes, and the optimally labeled conjugates of the invention are typically at least two-fold, usually more than three-fold and sometimes more than four-fold more fluorescent than conjugates of the Cy5 dye at the same antibody concentration (Table 3).

Table 3					
Protein	Source	Compound	DOS†	RQY†	TF§
GAR IgG	Molecular Probes	9	2.10	1.6	3.3
GAR IgG	Molecular Probes	9	3.0	1.4	4.3
GAR IgG	Molecular Probes	9	4.1	1.1	4.5
GAR IgG	Molecular Probes	9	5.2	0.9	4.7
GAR IgG	Molecular Probes	9	7.6	0.6	4.6
GAR IgG	Molecular Probes	9	8.2	0.5	4.1
GAR IgG	Molecular Probes	Cy5	2.4	0.95	2.3
GAR IgG	Molecular Probes	Cy5	4.1	0.5	2.1

GAR IgG	Molecular Probes	Cy5	4.5	0.2	0.9
GAR IgG	Molecular Probes	Cy5	5	0.3	1.5
GAR IgG	Molecular Probes	Cy5	5.8	0.03	0.2
GAR IgG	Jackson Labs	Cy5	2.2	1.1	2.4
GAR IgG	Chemicon	Cy5	3.3	0.82	2.7
GAR IgG	Zymed	Cy5	4.7	0.53	2.5
GAR IgG	Amersham-Pharmacia Biotech	Cy5	5.7	0.22	1.3
GAR IgG	Kirkegaard & Perry	Cy5	5.7	0.20	1.1
GAR IgG	Rockland	Cy5	10.1	0.05	0.5
GAR IgG	Molecular Probes	30	2.7	0.7	1.9
GAR IgG	Molecular Probes	30	4.3	0.28	1.2
GAR IgG	Molecular Probes	30	5.4	0.10	0.5
GAR IgG	Molecular Probes	24	2.9	0.8	2.2
GAR IgG	Molecular Probes	24	4.3	0.33	1.4
GAR IgG	Molecular Probes	24	5.6	0.15	0.8
GAR IgG	Molecular Probes	25	2.0	0.7	1.4
GAR IgG	Molecular Probes	25	2.9	0.35	1.0
GAR IgG	Molecular Probes	25	3.9	0.13	0.5
GAR IgG	Molecular Probes	27	2.0	0.9	1.8
GAR IgG	Molecular Probes	27	3.2	0.52	1.6
GAR IgG	Molecular Probes	27	4.2	0.28	1.2
GAR IgG	Molecular Probes	26	1.4	0.58	0.81
GAR IgG	Molecular Probes	26	2.1	0.3	0.61
GAR IgG	Molecular Probes	26	2.9	0.1	0.29
GAM IgG	Molecular Probes	9	2.1	1.4	2.9
GAM IgG	Molecular Probes	9	2.2	1.4	3.1
GAM IgG	Molecular Probes	9	3.1	1.1	3.5
GAM IgG	Molecular Probes	9	4.2	1.2	5.0
GAM IgG	Molecular Probes	9	5.2	0.6	3.2
GAM IgG	Jackson Labs	Cy5	1.9	1.0	1.9
GAM IgG	Molecular Probes	Cy5	2	0.9	1.8
GAM IgG	Molecular Probes	Cy5	3.3	0.5	1.6

GAM IgG	Molecular Probes	Cy5	4.8	0.09	0.4
Concanavalin A	Molecular Probes	9	1.7	1.2	2.0
Concanavalin A	Molecular Probes	9	2.2	0.8	1.8
Concanavalin A	Molecular Probes	9	3.3	0.9	2.9
Concanavalin A	Molecular Probes	9	3.7	0.6	2.3
Concanavalin A	Molecular Probes	9	5.5	0.6	3.3
Concanavalin A	Molecular Probes	9	5.6	0.8	4.5
Concanavalin A	Molecular Probes	Cy5	1.3	0.9	1.2
Concanavalin A	Molecular Probes	Cy5	2.4	0.5	1.2
Concanavalin A	Molecular Probes	Cy5	3.1	0.2	0.6
Concanavalin A	Molecular Probes	Cy5	3.3	0.8	2.6
Concanavalin A	Molecular Probes	Cy5	4.4	0.3	1.3
Concanavalin A	Molecular Probes	Cy5	6.5	0.1	0.7
Streptavidin	Molecular Probes	9	2.2	2.4	5.4
Streptavidin	Molecular Probes	9	2.8	1.9	5.3
Streptavidin	Molecular Probes	9	3.4	1.9	6.5
Streptavidin	Molecular Probes	9	4.1	1.8	7.3
Streptavidin	Molecular Probes	9	4.5	1.7	7.6
Streptavidin	Molecular Probes	9	5.2	1.6	8.3
Streptavidin	Molecular Probes	Cy5	1.6	1.8	2.8
Streptavidin	Molecular Probes	Cy5	2.7	1.5	4.1
Streptavidin	Jackson Labs	Cy5	3.3	1.8	6.0
Streptavidin	Amersham-Pharmacia Biotech	Cy5	3.6	1.4	5.0
Streptavidin	Molecular Probes	Cy5	3.6	1.4	5.0
Transferrin	Molecular Probes	9	1.8	1.5	2.7
Transferrin	Molecular Probes	9	2.7	1.3	3.5
Transferrin	Molecular Probes	9	3.7	1.2	4.4
Transferrin	Molecular Probes	9	4.2	1.1	4.4
Transferrin	Molecular Probes	9	5.8	0.7	4.1
Transferrin	Molecular Probes	9	5.9	0.6	3.5

Transferrin	Molecular Probes	Cy5	0.8	1.1	0.9
Transferrin	Molecular Probes	Cy5	1.4	0.8	1.1
Transferrin	Molecular Probes	Cy5	2.7	0.5	1.4
Transferrin	Molecular Probes	Cy5	3.0	0.7	2.1
Transferrin	Molecular Probes	Cy5	5.3	0.1	0.5
Transferrin	Molecular Probes	Cy5	5.8	0.02	0.1

†RQY (Relative Quantum Yield) is measured by matching absorbance between bioconjugate and DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridine-2-one)), and comparing integrated fluorescence emission intensities.

‡DOS (Degree of Substitution) is the approximate molar ratio of the dye to protein following conjugation. DOS estimated using Molar Extinction coefficients of 250,000 M⁻¹ cm⁻¹ for Cy5 and 239,000 M⁻¹ cm⁻¹ for Compound 9 bioconjugates respectively, and protein extinction coefficients from the literature and a visible dye-correction term (at 280 nm) of 3-5%.

§TF (Total Fluorescence) is proportional to the overall brightness of the bioconjugate, and is defined as the product of the RQY and DOS: TF= RQY x DOS.

Alternatively, Sc is a ligand or a hapten, such as biotin. A preferred conjugate is a phenol such as a tyramine (e.g. as described in U.S. Patents 5,196,306; 5,583,001; 5,731,158; all incorporated by reference), wherein the conjugate is useful as a substrate for horseradish peroxidase.

In one embodiment, Sc is a biological polymer such as a peptide, protein, oligonucleotide, or nucleic acid polymer that is also labeled with at least a second non-fluorescent or fluorescent dye (optionally an additional dye of the present invention), to form an energy-transfer pair. In some aspects of the invention, the labeled conjugate functions as an enzyme substrate, and enzymatic hydrolysis disrupts the energy transfer. Alternatively, Sc is itself a fluorescent or nonfluorescent dye, optionally an additional dye of the present invention, which dye-conjugate forms a labeling complex that exhibits a large Stokes shift due to internal energy-transfer (as described in U.S. Pat. 6,008,373 above), which complex is useful to label an organic or inorganic substance (Example 52).

In one embodiment, Sc is an amino acid (including those that are protected or are substituted by phosphates, carbohydrates, or C₁ to C₂₃ carboxylic acids), or is a polymer of amino acids such as a peptide or protein. Preferred conjugates of peptides contain at least five amino acids, more preferably 5 to 36 amino acids. Preferred

peptides include, but are not limited to, neuropeptides, cytokines, toxins, protease substrates, and protein kinase substrates. Preferred protein conjugates include enzymes, antibodies, lectins, glycoproteins, histones, albumins, lipoproteins, avidin, streptavidin, protein A, protein G, phycobiliproteins and other fluorescent proteins,

5 hormones, toxins, chemokines and growth factors. In one preferred aspect, the conjugated protein is a phycobiliprotein, such as allophycocyanin, phycocyanin, phycoerythrin, allophycocyanin B, B-phycoerythrin, phycoerythrocyanin, and b-phycoerythrin (for example, see U.S. Pat. 5,714,386 to Roederer (1998), incorporated by reference). Particularly preferred are conjugates of R-phycoerythrin and of

10 allophycocyanin with selected dyes of the invention that serve as excited-state energy acceptors or donors. In these conjugates, excited state energy transfer results in long wavelength fluorescence emission when excited at relatively short wavelengths (Example 52). In another aspect of the invention, the conjugated protein is an antibody, an antibody fragment, avidin, streptavidin, a toxin, a lectin, a hormone, a

15 chemokine, or a growth factor. Typically, where the conjugated substance is a toxin, it is a neuropeptide or a phallotoxin, such as phalloidin.

In another embodiment, S_0 is a nucleic acid base, nucleoside, nucleotide (Example 55) or a nucleic acid polymer (Examples 56-59), including those that are

20 modified to possess an additional linker or spacer for attachment of the dyes of the invention, such as an alkynyl linkage (U.S. Pat. 5,047,519), an aminoalkyl linkage (U.S. Pat. 4,711,955), or a heteroatom-substituted linker (U.S. Pat. 5,684,142) (all patents incorporated by reference), or other linkage. In another embodiment, the conjugated substance is a nucleoside or nucleotide analog that links a purine or

25 pyrimidine base to a phosphate or polyphosphate moiety through a noncyclic spacer. In a third embodiment, the dye is conjugated to the carbohydrate portion of a nucleotide or nucleoside, typically through a hydroxyl group but additionally through a thiol or amino group (U.S. Pat. 5,659,025, U.S. Pat. 5,668,268, U.S. Pat. 5,679,785; all incorporated by reference). Typically, the conjugated nucleotide is a nucleoside

30 triphosphate or a deoxynucleoside triphosphate or a dideoxynucleoside triphosphate. Incorporation of methylene moieties or nitrogen or sulfur heteroatoms into the phosphate or polyphosphate moiety is also useful. Nonpurine and nonpyrimidine bases such as 7-deazapurines (US Pat. 6,150,510, incorporate by reference) and nucleic acids containing such bases can also be coupled to dyes of the invention.

Nucleic acid adducts prepared by reaction of depurinated nucleic acids with amine, hydrazide or hydroxylamine derivatives provide an additional means of labeling and detecting nucleic acids, e.g. "A method for detecting abasic sites in living cells: age-dependent changes in base excision repair." Atamna H, Cheung I, Ames BN. Proc Natl Acad Sci U S A 97, 686-691 (2000); incorporated by reference.

Preferred nucleic acid polymer conjugates are labeled, single- or multi-stranded, natural or synthetic DNA or RNA, DNA or RNA oligonucleotides, or DNA/RNA hybrids, or incorporate an unusual linker such as morpholine derivatized phosphates (AntiVirals, Inc., Corvallis OR), or peptide nucleic acids such as *N*-(2-aminoethyl)glycine units. When the nucleic acid is a synthetic oligonucleotide, it typically contains fewer than 50 nucleotides, more typically fewer than 25 nucleotides. Conjugates of peptide nucleic acids (PNA) (Nielsen et al U.S. Pat. 5,539,082, incorporated by reference) may be preferred for some applications because of their generally faster hybridization rates.

Fluorescent nucleic acid polymers are typically prepared from labeled nucleotides or oligonucleotides using oligonucleotide-primed DNA polymerization (Example 59), such as by using the polymerase chain reaction or through primer extension, or by terminal-transferase catalyzed addition of a labeled nucleotide to a 3'-end of a nucleic acid polymer. Fluorescent RNA polymers are typically prepared from labeled nucleotides by transcription. Typically, the dye is attached via one or more purine or pyrimidine bases through an amide, ester, ether or thioether bond; or is attached to the phosphate or carbohydrate by a bond that is an ester, thioester, amide, ether or thioether. Alternatively, dye conjugate of the invention is simultaneously labeled with a hapten such as biotin or digoxigenin, or to an enzyme such as alkaline phosphatase, or to a protein such as an antibody. Nucleotide conjugates of the invention are readily incorporated by DNA polymerase and can be used for *in situ* hybridization and nucleic acid sequencing (e.g., U.S. Patents 5,332,666; 5,171,534; and 4,997,928, and WO Appl. 94/05688; all incorporated by reference). In another aspect of the invention, the oligonucleotide incorporates an aliphatic amine, which is subsequently conjugated to an amine-reactive dye of the invention or a thiol or thiophosphate, which is conjugated to a thiol-reactive dye of the invention. In yet another aspect of the invention, the purine bases of the

oligonucleotide react with a reactive metal complex (preferably a platinum complex) bound to a dye of the invention, yielding a dye-conjugate (Example 58). Nucleic acid conjugates of dyes of the invention that are linked at the 3-position of the indolium ring unexpectedly have spectral properties that are superior to those of structurally similar carbocyanine dyes wherein the dye is not linked at the 3-position of the indolium ring (Examples 58-60, Table 8).

In one embodiment, the conjugated oligonucleotides of the invention are aptamers for a particular target molecule, such as a metabolite, dye, hapten, or protein. That is, the oligonucleotides have been selected to bind preferentially to the target molecule. Methods of preparing and screening aptamers for a given target molecule have been previously described and are known in the art (for example, U.S. Pat. 5,567,588 to Gold (1996), incorporated by reference).

In another embodiment, the conjugated substance (S_c) is a carbohydrate that is typically a polysaccharide, such as a dextran, FICOLL™, heparin, glycogen, amylopectin, mannan, inulin, starch, agarose and cellulose. Alternatively, the carbohydrate is a polysaccharide that is a lipopolysaccharide. Preferred polysaccharide conjugates are dextran, FICOLL™, or lipopolysaccharide conjugates.

In another embodiment, the conjugated substance (S_c), is a lipid (typically having 6–60 carbons), including glycolipids, phospholipids, sphingolipids, and steroids. Alternatively, the conjugated substance is a lipid assembly, such as a liposome. The lipophilic moiety may be used to retain the conjugated substances in cells, as described in US Pat. 5,208,148 (incorporated by reference). Certain polar dyes of the invention may also be trapped within lipid assemblies.

Conjugates having an ion-complexing moiety serve as indicators for calcium, sodium, magnesium, zinc, potassium, or other biologically important metal ions. Preferred ion-complexing moieties are crown ethers (U.S. Pat. 5,405,975 and provisional patent application titled "Crown Ether Derivatives", filed December 19, 2000 by Martin et al. US S/N 60/258,266, both incorporated by reference); derivatives of 1,2-bis-(2-aminophenoxyethane)- N,N,N',N' -tetraacetic acid (BAPTA chelators; U.S. Pat. 5,453,517, U.S. Pat. 5,516,911, and U.S. Pat. 5,049,673, all incorporated by

reference); derivatives of 2-carboxymethoxyaniline-N,N-diacetic acid (APTRA chelators; AM. J. PHYSIOL. 256, C540 (1989), incorporated by reference); or pyridine- and phenanthroline-based metal ion chelators (U.S. Pat. 5,648,270, incorporated by reference); or derivatives of nitrilotriacetic acid, see e.g. "Single-step
5 synthesis and characterization of biotinylated nitrilotriacetic acid, a unique reagent for the detection of histidine-tagged proteins immobilized on nitrocellulose", McMahan SA, Burgess RR. Anal Biochem 236, 101-106 (1996); incorporated by reference. Preferably, the ion-complexing moiety is a crown ether chelator, a BAPTA chelator, an APTRA chelator or a derivative of nitrilotriacetic acid.

10

Other conjugates of non-biological materials include dye-conjugates of organic or inorganic polymers, polymeric films, polymeric wafers, polymeric membranes, polymeric particles, or polymeric microparticles (Example 53); including magnetic and non-magnetic microspheres; iron, gold or silver particles; conducting and non-
15 conducting metals and non-metals; and glass and plastic surfaces and particles. Conjugates are optionally prepared by copolymerization of a dye that contains an appropriate functionality while preparing the polymer, or by chemical modification of a polymer that contains functional groups with suitable chemical reactivity. Other types of reactions that are useful for preparing dye-conjugates of polymers include
20 catalyzed polymerizations or copolymerizations of alkenes and reactions of dienes with dienophiles, transesterifications or transaminations. In another embodiment, the conjugated substance is a glass or silica, which may be formed into an optical fiber or other structure.

25

In one aspect of the invention, S_c is a conjugated substance that is an antibody (including intact antibodies, antibody fragments, and antibody sera, etc.), an amino acid, an angiostatin or endostatin, an avidin or streptavidin, a biotin (e.g. an amidobiotin, a biocytin, a desthiobiotin, etc.), a blood component protein (e.g. an albumin, a fibrinogen, a plasminogen, etc.), a dextran, an enzyme, an enzyme
30 inhibitor, an IgG-binding protein (e.g. a protein A, protein G, protein A/G, etc.), a fluorescent protein (e.g. a phycobiliprotein, an aequorin, a green fluorescent protein, etc.), a growth factor, a hormone, a lectin (e.g. a wheat germ agglutinin, a concanavalin A, etc.), a lipopolysaccharide, a metal-binding protein (e.g. a calmodulin, etc.), a microorganism or portion thereof (e.g. a bacteria, a virus, a yeast, etc.), a

neuropeptide and other biologically active factors (e.g. a dermorphin, a deltorphin, an endomorphin, an endorphin, a tumor necrosis factor etc.), a non-biological microparticle (e.g. of ferrofluid, gold, polystyrene, etc.), a nucleotide, an oligonucleotide, a peptide toxin (e.g. an apamin, a bungarotoxin, a phalloidin, etc.), a
5 phospholipid-binding protein (e.g. an annexin, etc.), a small-molecule drug (e.g. a methotrexate, etc.), a structural protein (e.g. an actin, a fibronectin, a laminin, a microtubule-associated protein, a tublin, etc.), or a tyramide.

In one embodiment, conjugates of biological polymers such as peptides,
10 proteins, oligonucleotides, nucleic acid polymers are also labeled with at least a second fluorescent or nonfluorescent dye, that is optionally an additional dye of the present invention, to form an energy-transfer pair. In some aspects of the invention, the labeled conjugate functions as an enzyme substrate, and enzymatic hydrolysis disrupts the energy transfer. Alternatively, the conjugated substance is itself a
15 fluorescent or nonfluorescent dye, optionally an additional dye of the present invention, that forms a labeling complex that exhibits a large Stokes shift due to internal energy-transfer (as described in U.S. Pat. 6,008,373 to Waggoner et al., (1999), incorporated by reference). In another embodiment of the invention, the energy-transfer pair that incorporates a dye of the invention is conjugated to an
20 oligonucleotide that displays efficient fluorescence quenching in its hairpin conformation (the so-called "molecular beacons" of Tyagi et al., NATURE BIOTECHNOLOGY 16, 49 (1998) incorporated by reference) or fluorescence energy transfer.

25 The preparation of dye conjugates using reactive dyes is well documented, e.g. by R. Haugland, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, Chapters 1-3 (1996); and Brinkley, BIOCONJUGATE CHEM., 3, 2 (1992). Conjugates typically result from mixing appropriate reactive dyes and the substance to be conjugated in a suitable solvent in
30 which both are soluble. The majority of the dyes of the invention are readily soluble in aqueous solutions, facilitating conjugation reactions with most biological materials. For those reactive dyes that are photoactivated, conjugation requires illumination of the reaction mixture to activate the reactive dye.

1. Labeled members of a specific binding pair are typically used as fluorescent probes for the complementary member of that specific binding pair, each specific binding pair member having a area on the surface or in a cavity that specifically binds to and is complementary with a particular spatial and polar organization of the other. Preferred specific binding pair members are proteins that bind non-covalently to low molecular weight ligands, such as biotin, drug-haptens and fluorescent dyes (such as an anti-fluorescein antibody). Representative specific binding pairs are shown in Table 4.

10 Table 4. Representative Specific Binding Pairs

antigen	antibody
biotin	avidin (or streptavidin or anti-biotin)
IgG*	protein A or protein G
drug	drug receptor
toxin	toxin receptor
carbohydrate	lectin or carbohydrate receptor
peptide	peptide receptor
protein	protein receptor
enzyme substrate	enzyme
DNA (RNA)	aDNA (aRNA)†
hormone	hormone receptor
ion	chelator
psoralen	nucleic acid
target molecule	RNA or DNA aptamer

* IgG is an immunoglobulin

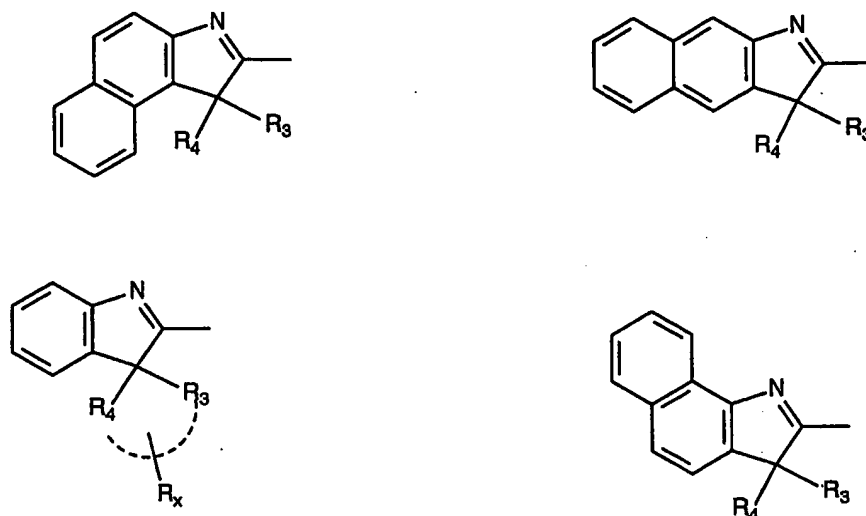
† aDNA and aRNA are the antisense (complementary) strands used for hybridization

- 15 In one aspect of the invention, the conjugated substance is further labeled with additional dye moieties, such that fluorescence energy is either accepted from, or transferred to, the dye of the invention.

Synthesis

Synthesis of the carbocyanine dyes of the invention, where attachment is at the 3-position of the indolium, depends on initial preparation of certain key intermediates. The intermediates have the following general structure (for simplicity, all but a few of the possible substituents are shown as hydrogen):

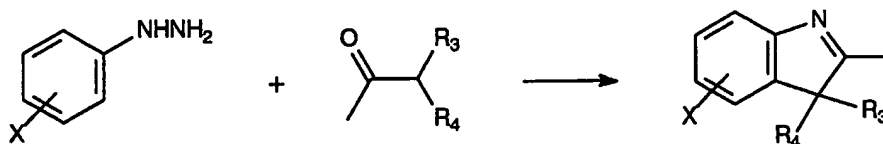
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- 10 These basic structures are optionally further substituted, during or after synthesis, to give the corresponding dye substituents as defined above.

- The novel key intermediates are readily synthesized by a reaction that is analogous to a Fischer indole synthesis (where X is a desired substituent on the resulting indolium, typically sulfo, and R³ and R⁴ are as defined above):

15



- In this reaction, an appropriately substituted aryl hydrazine, which is typically a phenylhydrazine or an appropriately substituted naphthyl hydrazine, is reacted with an appropriately substituted methyl ketone to yield a 3,3-disubstituted 2-methylindole derivative. One of the 3-position substituents is selected to be a

20

chemically reactive moiety or a group that is converted to a chemically reactive moiety such as a carboxylic acid derivative (Examples 1, 2, 7), an alcohol (Example 16) or an amine (Example 17). It is particularly suitable to utilize a sulfonated phenylhydrazine derivative (as in Examples 1-3) or a sulfonated naphthylhydrazine derivative (as in Example 25) to increase the solubility of the final dye. The 3,3-disubstituted-2-methylindole is then quaternized on the nitrogen atom to an indolium derivative with an alkylating agent that is typically an alkyl halide such as ethyl iodide, an alkylsulfonate such as methyl p-toluenesulfonate (Example 7) or a cyclic sulfonate such as propanesultone or butanesultone (Examples 2-3). Typically, the key indolium or benzindolium intermediates are sulfonated one or more times before or after quaternization at R² and subsequent condensation with the benzazolum moiety and polymethine moiety to form the subject dyes. Methods for synthesis of dyes wherein n = 1, n = 2 and n = 3 are provided in Examples 12, 8, and 21, respectively. Variations on these methods are well known in the art that yield substituents on the polymethine bridge or on the indolium or benzolium portion of the dye precursor.

A useful synthetic route to the azacarbocyanine dyes of the present invention can be described in three parts, following the natural breakdown in the description of the compounds. In general, the synthesis of these dyes requires three precursors: the appropriate benzazolum or azabenzazolum salt (the "A" and "B" moieties), and a source for the polymethine spacer. Typically each component is selected so as to incorporate the appropriate chemical substituents, or functional groups that can be converted to the appropriate substituents. The chemistry that is required to prepare and combine these precursors so as to yield any of the subject derivatives is generally well understood by one skilled in the art. Although there are many possible variations that may yield an equivalent result, we provide herein some useful general methods for their synthesis and incorporation of chemical modifications.

Although the chemically reactive azabenzazolum dyes and their conjugates described herein have not previously been described, a variety of nonreactive azabenzazolum derivatives have been previously described (see, for example, Brooker, et al., J. AM. CHEM. SOC., 64, 199 (1942); Heravi, et al., INDIAN J. CHEM. 36B, 1025 (1997); Smith et al. SULFUR LETTERS 17, 197 (1994); Chu-Moyer et al. J. ORG. CHEM. 60, 5721 (1995); Turner, J. ORG. CHEM. 48, 3401

(1983); Couture et al. J. HETEROCYCLIC CHEM. 24, 1765 (1987); Petric et al. J. HETEROCYCLIC CHEM. 14, 1045, (1977); Barlin et al. AUST. J. CHEM., 37, 1729 (1984); Saikachi et al. CHEM. & PHARM. BULL. 9, 941 (1961); Barlin AUST. J. CHEM. 36, 983 (1983); Foye et al., J. PHARM. SCI. 64, 1371 (1975); Khanna et al. J. 5 ORG. CHEM. 60, 960 (1995)); British Patent No. 870,753 to Ficken et al. (1961); Ficken et al., "Diazaindenes and Their Quaternary Salts-Part I" pp 3202-3212 (1959); Ficken et al., "Diazaindenes and Their Quaternary Salts-Part II" pp 584-588 (1961). Synthetic methods for preparing some azabenzazolum precursors have also been described in copending application serial no. 09/557,275 by Haugland et al., filed 10 April 24, 2000, hereby incorporated by reference.

The substituents on the aromatic carbons of the azabenzazolum moiety are typically incorporated in the parent aza- or polyazabenzazole molecule prior to quaternization with an alkylating agent. However, such substituents may also be 15 incorporated during the synthesis of the azabenzazole moiety. R² / R¹² is usually obtained by alkylation of the parent heterocycle with an alkylating agent that incorporates the desired R² / R¹² moiety.

The B moiety intermediate is optionally an azabenzazolum precursor, as 20 described above, or is a benzazolum precursor, as well known in the art (for example, U.S. Patent No. 5,436,134 to Haugland et al., (1995), incorporated by reference). The B moiety is optionally fused to additional rings, resulting in dyes that absorb and emit at longer wavelengths (for example, see U.S. 6,027,709 to Little et al. (2000), incorporated by reference).

25

Alkyl, alkoxy, carboxyl, and halogen substituents at aromatic carbons are typically already present as substituents on the benzazole or azabenzazole precursors, or on compounds that are readily converted to such precursors using methods well-known in the art. Sulfonic acid groups are typically introduced on the 30 precursors prior to condensation of the cyanine dye (for example, see U.S. 5,767,287 to Bobrow et al. (1998), incorporated by reference). Aminoalkyl groups are typically substituted by a protecting group when they are first introduced, typically by substitution onto the benzazole or azabenzazole precursor. The protecting group is then removed after condensation of the cyanine dye. Aromatic amino groups are

typically prepared via the reduction of a nitro substituted benzazolium precursor, which in turn is prepared by the nitration of the benzazole precursor.

The BRIDGE moiety typically originates from the coupling agent used in the dye construction. For example, N,N'-diphenylformamidine and triethylorthoformate yields BRIDGE moieties wherein a and b are both zero. Malonaldehyde bis(phenylimine) hydrochloride, 1,1,3-trimethoxypropane, and 1,1,3,3-tetramethoxypropane yield dyes wherein one of a and b is 1, and glutacanaldehyde dianil monochloride yields dyes wherein both a and b are 1.

The methods for synthesis of dyes that contain a variety of reactive groups such as those described in Table 2 are well documented in the art. Particularly useful are amine-reactive dyes such as "activated esters" of carboxylic acids, which are typically synthesized by coupling a carboxylic acid to a relatively acidic "leaving group". Other preferred amine-reactive groups include sulfonyl halides, which are prepared from sulfonic acids using a halogenating agent such as PCl₅ or POCl₃; halotriazines, which are prepared by the reaction of cyanuric halides with amines; and isothiocyanates or isothiocyanates, which are prepared from amines and phosgene or thiophosgene, respectively.

Dyes containing amines and hydrazides are particularly useful for conjugation to carboxylic acids, aldehydes and ketones. Most often these are synthesized by reaction of an activated ester of a carboxylic acid or a sulfonyl halide with a diamine, such as cadaverine, or with a hydrazine. Alternatively, aromatic amines are commonly synthesized by chemical reduction of a nitroaromatic compound. Amines and hydrazines are particularly useful precursors for synthesis of thiol-reactive haloacetamides or maleimides by standard methods.

Nucleosides and nucleotides labeled with dyes of the invention are particularly useful for some applications of nucleic acid labeling. The use of carbocyanine-amidites for labeling nucleotides and nucleosides have been previously described (U.S. 5,986,086 to Bruch et al. (1999); U.S. 5,808,044 to Brush et al. (1998); U.S. 5,556,959 to Brush et al. (1996); all incorporated by reference).

Examples of some synthetic strategies for selected dyes of the invention, as well as their characterization, synthetic precursors, conjugates and method of use are provided in the examples below. Further modifications and permutations will be obvious to one skilled in the art.

5

Applications and Methods of Use

In one aspect of the invention, the dye compounds of the invention are used to directly stain or label a sample so that the sample can be identified or quantitated.

- 10 For instance, such dyes may be added as part of an assay for a biological target analyte, as a detectable tracer element in a biological or non-biological fluid, or for such purposes as photodynamic therapy of tumors, in which a dyed sample is irradiated to selectively destroy tumor cells and tissues, or to photoablate arterial plaque or cells, usually through the photosensitized production of singlet oxygen. In
- 15 one preferred embodiment, dye conjugate is used to stain a sample that comprises a ligand for which the conjugated substance is a complementary member of a specific binding pair (e.g. Table 4).

- In one aspect of the invention, the sample is obtained directly from a liquid
- 20 source or as a wash from a solid material (organic or inorganic) or a growth medium in which cells have been introduced for culturing, or a buffer solution in which cells have been placed for evaluation. Where the sample comprises cells, the cells are optionally single cells, including microorganisms, or multiple cells associated with other cells in two or three dimensional layers, including multicellular organisms,
- 25 embryos, tissues, biopsies, filaments, biofilms, etc.

- Alternatively, the sample is a solid, optionally a smear or scrape or a retentate removed from a liquid or vapor by filtration. In one aspect of the invention, the sample is obtained from a biological fluid, including separated or unfiltered biological
- 30 fluids such as urine, cerebrospinal fluid, blood, lymph fluids, tissue homogenate, interstitial fluid, cell extracts, mucus, saliva, sputum, stool, physiological secretions or other similar fluids. Alternatively, the sample is obtained from an environmental source such as soil, water, or air; or from an industrial source such as taken from a waste stream, a water source, a supply line, or a production lot.

In yet another embodiment, the sample is present on or in solid or semi-solid matrix. In one aspect of the invention, the matrix is a membrane. In another aspect, the matrix is an electrophoretic gel, such as is used for separating and characterizing nucleic acids or proteins, or is a blot prepared by transfer from an electrophoretic gel to a membrane. In another aspect, the matrix is a silicon chip or glass slide, and the analyte of interest has been immobilized on the chip or slide in an array (e.g. the sample comprises proteins or nucleic acid polymers in a microarray). In yet another aspect, the matrix is a microwell plate or microfluidic chip, and the sample is analyzed by automated methods, typically by various methods of high-throughput screening, such as drug screening.

The dye compounds of the invention are generally utilized by combining a dye compound of the invention as described above with the sample of interest under conditions selected to yield a detectable optical response. The term "dye compound" is used herein to refer to all aspects of the claimed dyes, including both reactive dyes and dye conjugates. The dye compound typically forms a covalent or non-covalent association or complex with an element of the sample, or is simply present within the bounds of the sample or portion of the sample. The sample is then illuminated at a wavelength selected to elicit the optical response. Typically, staining the sample is used to determine a specified characteristic of the sample by further comparing the optical response with a standard or expected response.

A detectable optical response means a change in, or occurrence of, an optical signal that is detectable either by observation or instrumentally. Typically the detectable response is a change in fluorescence, such as a change in the intensity, excitation or emission wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof. The degree and/or location of staining, compared with a standard or expected response, indicates whether and to what degree the sample possesses a given characteristic. Some dyes of the invention may exhibit little fluorescence emission, but are still useful as chromophoric dyes. Such chromophores are useful as energy acceptors in FRET applications, or to simply impart the desired color to a sample or portion of a sample.

For biological applications, the dye compounds of the invention are typically used in an aqueous, mostly aqueous or aqueous-miscible solution prepared according to methods generally known in the art. The exact concentration of dye compound is dependent upon the experimental conditions and the desired results, but typically
5 ranges from about one nanomolar to one millimolar or more. The optimal concentration is determined by systematic variation until satisfactory results with minimal background fluorescence is accomplished.

The dye compounds are most advantageously used to stain samples with
10 biological components. The sample may comprise heterogeneous mixtures of components (including intact cells, cell extracts, bacteria, viruses, organelles, and mixtures thereof), or a single component or homogeneous group of components (e.g. natural or synthetic amino acid, nucleic acid or carbohydrate polymers, or lipid membrane complexes). These dyes are generally non-toxic to living cells and other
15 biological components, within the concentrations of use.

The dye compound is combined with the sample in any way that facilitates contact between the dye compound and the sample components of interest. Typically, the dye compound or a solution containing the dye compound is simply added to the
20 sample. Certain dyes of the invention, particularly those that are substituted by one or more sulfonic acid moieties, tend to be impermeant to membranes of biological cells, and once inside viable cells are typically well retained. Treatments that permeabilize the plasma membrane, such as electroporation, shock treatments or high extracellular ATP can be used to introduce selected dye compounds into cells.
25 Alternatively, selected dye compounds can be physically inserted into cells, e.g. by pressure microinjection, scrape loading, patch clamp methods, or phagocytosis.

Dyes that incorporate an aliphatic amine or a hydrazine residue can be microinjected into cells, where they can be fixed in place by aldehyde fixatives such as
30 formaldehyde or glutaraldehyde. This fixability makes such dyes useful for intracellular applications such as neuronal tracing.

Dye compounds that possess a lipophilic substituent, such as phospholipids, will non-covalently incorporate into lipid assemblies, e.g. for use as probes for

membrane structure; or for incorporation in liposomes, lipoproteins, films, plastics, lipophilic microspheres or similar materials; or for tracing. Lipophilic dyes are useful as fluorescent probes of membrane structure.

5 Chemically reactive dye compounds will covalently attach to a corresponding functional group on a wide variety of materials, forming dye conjugates as described above. Using dye compounds to label reactive sites on the surface of cells, in cell membranes or in intracellular compartments such as organelles, or in the cell's cytoplasm, permits the determination of their presence or quantity, accessibility, or
10 their spatial and temporal distribution in the sample. Photoreactive dyes can be used similarly to photolabel components of the outer membrane of biological cells or as photo-fixable polar tracers for cells.

 Optionally, the sample is washed after staining to remove residual, excess or
15 unbound dye compound. The sample is optionally combined with one or more other solutions in the course of staining, including wash solutions, permeabilization and/or fixation solutions, and solutions containing additional detection reagents. An additional detection reagent typically produces a detectable response due to the presence of a specific cell component, intracellular substance, or cellular condition,
20 according to methods generally known in the art. Where the additional detection reagent has, or yields a product with, spectral properties that differ from those of the subject dye compounds, multi-color applications are possible. This is particularly useful where the additional detection reagent is a dye or dye-conjugate of the present invention having spectral properties that are detectably distinct from those of the
25 staining dye.

 The compounds of the invention that are dye conjugates are used according to methods extensively known in the art; e.g. use of antibody conjugates in microscopy and immunofluorescent assays; and nucleotide or oligonucleotide conjugates for
30 nucleic acid hybridization assays and nucleic acid sequencing (e.g., US Patent Nos. 5,332,666 to Prober, et al. (1994); 5,171,534 to Smith, et al. (1992); 4,997,928 to Hobbs (1991); and WO Appl. 94/05688 to Menchen, et al.; all incorporated by reference). Dye-conjugates of multiple independent dyes of the invention possess utility for multi-color applications.

At any time after or during staining, the sample is illuminated with a wavelength of light selected to give a detectable optical response, and observed with a means for detecting the optical response. Equipment that is useful for illuminating the dye compounds of the invention includes, but is not limited to, hand-held ultraviolet lamps, mercury arc lamps, xenon lamps, lasers and laser diodes. These illumination sources are optionally integrated into laser scanners, fluorescence microplate readers, standard or minifluorometers, or chromatographic detectors. Preferred embodiments of the invention are dyes that are be excitable at or near the wavelengths 633-636 nm, 647 nm, 660 nm, 680 nm and beyond 700 nm, as these regions closely match the output of relatively inexpensive excitation sources.

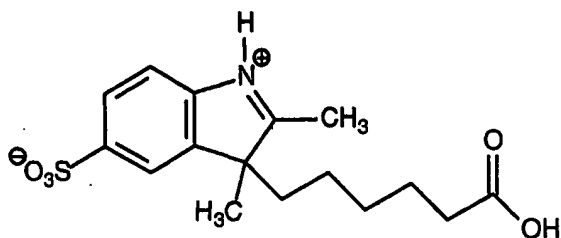
The optical response is optionally detected by visual inspection, or by use of any of the following devices: CCD cameras, video cameras, photographic film, laser-scanning devices, fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or by means for amplifying the signal such as photomultiplier tubes. Where the sample is examined using a flow cytometer, examination of the sample optionally includes sorting portions of the sample according to their fluorescence response.

Kits

One aspect of the instant invention is the formulation of kits that facilitate the practice of various assays using any of the dyes of the invention, as described above. The kits of the invention typically comprise a colored or fluorescent dye of the invention, either present as a chemically reactive label useful for preparing dye-conjugates, or present as a dye-conjugate where the conjugated substance is a specific binding pair member, or a nucleoside, nucleotide, oligonucleotide, nucleic acid polymer, peptide, or protein. The kit optionally further comprises one or more buffering agents, typically present as an aqueous solution. The kits of the invention optionally further comprise additional detection reagents, a purification medium for purifying the resulting labeled substance, luminescence standards, enzymes, enzyme inhibitors, organic solvent, or instructions for carrying out an assay of the invention.

The examples below are given so as to illustrate the practice of this invention.
They are not intended to limit or define the entire scope of this invention.

Example 1. Preparation of 3-(5-carboxypentyl)-2,3-dimethyl-5-sulfoindolium, inner salt (Compound 1).

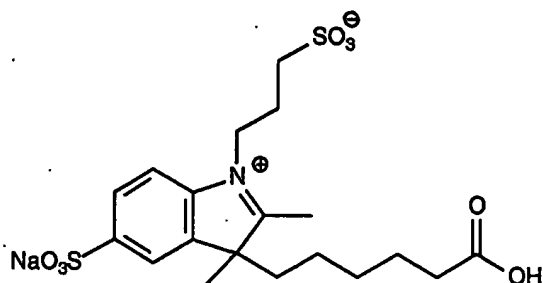


Compound 1

- 5 A mixture of 25 g of ethyl 2-methylacetoacetate, 64 mL of a 21% sodium ethoxide solution in ethanol and 34 mL of ethyl 6-bromohexanoate is refluxed in 200 mL of ethanol overnight. The mixture is filtered and solvent is evaporated. The residue is partitioned between 1 M HCl and chloroform. The organic layer is dried over magnesium sulfate and purified on silica gel using 1:10 ethyl acetate/ hexanes as eluant to yield 22 g of ethyl 2-(5-carboxypentyl)-2-methylacetoacetate.
- 10

- The acetoacetate thus obtained is dissolved in 300 mL of methanol. A solution of 10 g NaOH in 100 mL water is added. The mixture is heated at 50°C overnight. The solution is reduced to ~50 mL, acidified to ~pH 1, and extracted with ethyl acetate. The organic layer is dried over MgSO₄ and evaporated to yield 13.5 g of 7-methyl-8-oxononanoic acid. The nonanoic acid is refluxed in 110 mL of acetic acid with 13.5 g of 4-hydrazinobenzenesulfonic acid for 5 hours. The acetic acid is evaporated and the product is purified on silica gel to yield 23 g of the product.
- 15

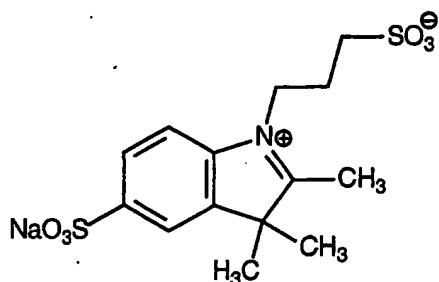
- 20 Example 2. Preparation of 2,3-dimethyl-3-(5-carboxypentyl)-5-sulfo-1-(3-sulfopropyl)indolium, sodium salt (Compound 2).



Compound 2

To a methanol solution of 11 g of Compound 1 is added 3.4 g of anhydrous sodium acetate. The mixture is stirred for five minutes. The solvent is evaporated. The resulting sodium salt is heated with 24.4 g of propanesultone at 110°C for 1 hour to generate the product.

Example 3. Preparation of 5-sulfo-1-(3-sulfopropyl)-2,3,3-trimethylindolium, sodium salt (Compound 3A) and 5-sulfo-1-(3-sulfopropyl)-1,2,3,3-tetramethylindolium, sodium salt (Compound 3B).

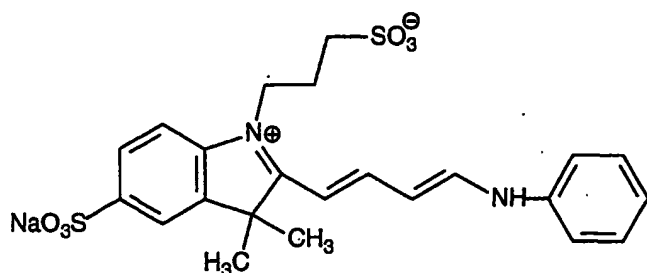


Compound 3A

To 15 g of 5-sulfo-2,3,3-trimethylindolium, inner salt (Mujumdar, et al BIOCONJUGATE CHEMISTRY 4, 105 (1993)) in 60 mL of methanol is added 5.67 g sodium acetate. After 5 minutes at room temperature, the solution is evaporated. The foamy solid is pulverized, dissolved in 60 mL acetonitrile and stirred with 23 g propanesultone for 15 min. Following evaporation of the solvent, the residue is dried at 110°C to yield 5-sulfo-1-(3-sulfopropyl)-2,3,3-trimethylindolium, sodium salt (Compound 3A). 5-sulfo-1,2,3,3-tetramethylindolium (Compound 3B) is prepared similarly except that methyl *p*-toluenesulfonate is used instead of propanesultone.

Example 4. Preparation of 2-(4-anilinobutadienyl)-3,3-dimethyl-5-sulfo-1-(3-sulfopropyl)indolium, sodium salt (Compound 4).

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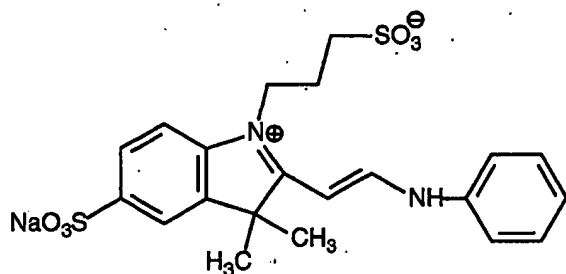
Compound 4

Compound 3A (15 g) is heated with 21.5 g malonaldehyde dianil hydrochloride and 0.4 mL triethylamine in 200 mL of acetic acid at 110°C for one hour. The solvent is evaporated and the residue is purified on silica gel to yield 1.39 g of the product.

10

Example 5. Preparation of 2-(anilinovinyl)-3,3-dimethyl-5-sulfo-1-(3-sulfopropyl)indolium, sodium salt (Compound 5).

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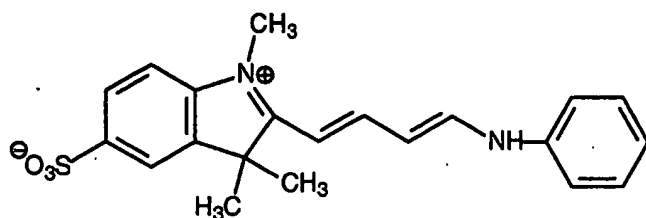


Compound 5

A mixture of 5 g of Compound 3A, 2.72 g of N,N'-diphenylformamidine and 0.52 mL of acetic anhydride is heated at 150°C for 30 minutes, then evaporated and the residue purified on silica gel.

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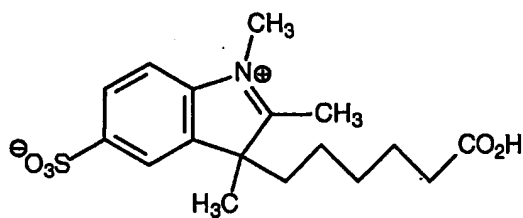
Example 6. Preparation of 2-(4-anilinobutadienyl)-5-sulfo-1,3,3-trimethylindolium, inner salt (Compound 6).



Compound 6

The procedure is the same as used to prepare Compound 4, except that Compound 3B is
5 used instead of Compound 3A.

Example 7. Preparation of 3-(5-carboxypentyl)-5-sulfo-1,2,3-trimethylindolium, inner salt (Compound 7).



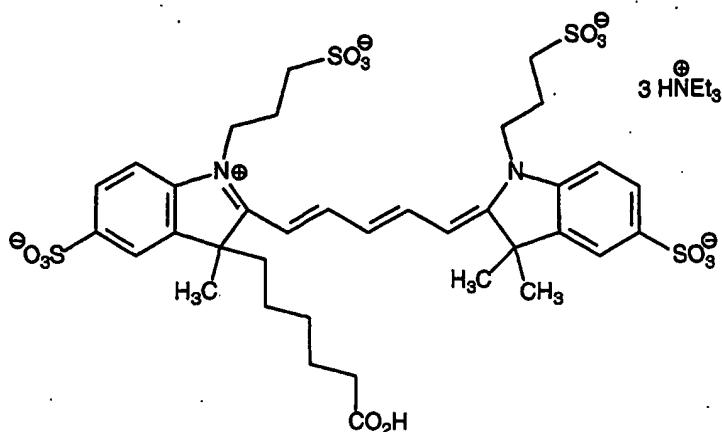
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Compound 7

The compound is prepared by heating Compound 1 with 6 equivalents of methyl p-toluenesulfonate at 100°C for 1.5 hours. The crude product is precipitated with ethyl acetate and purified on a silica gel column.

15

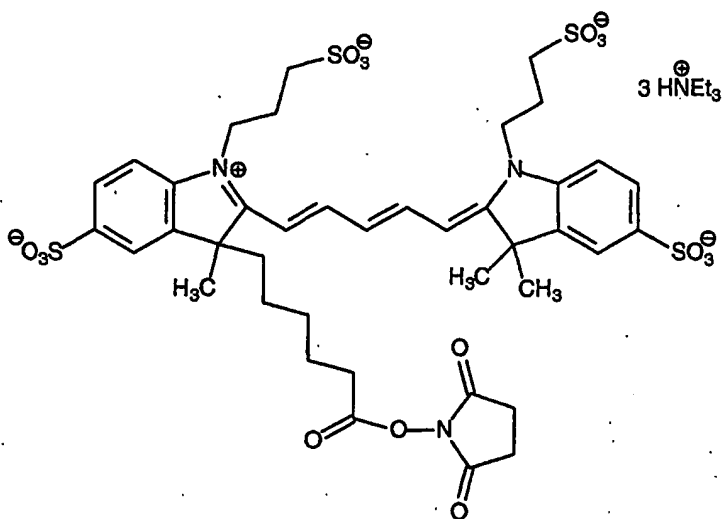
Example 8. Preparation of Compound 8.



Compound 8

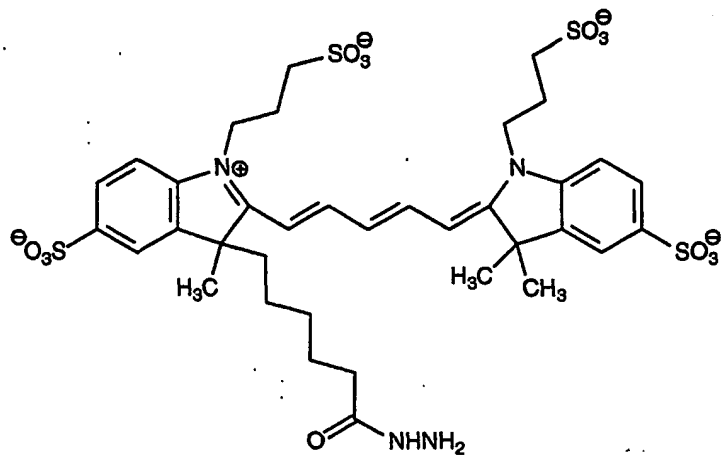
Compound 2 (1 g) and Compound 4 (1.5 g) are combined with 0.84 mL triethylamine and 0.5 mL acetic anhydride. The mixture is stirred at room temperature for 1 hour, then evaporated and the residue is purified by HPLC.

Example 9. Preparation of Compound 9.



Compound 9

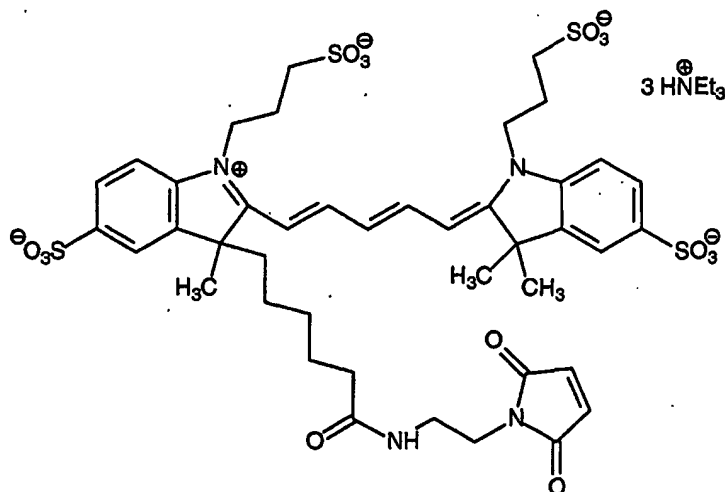
To 55 mg of Compound 8 in 1 mL of DMF is added 0.034 mL of triethylamine and 21 mg of 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate. The mixture is stirred at room temperature for 30 minutes and evaporated to yield the succinimidyl ester.

Example 10. Preparation of Compound 10.**Compound 10**

5

To Compound 9 in acetonitrile is added 3 equivalents of triethylamine and 1.2 equivalents anhydrous hydrazine. The mixture is stirred at ambient temperature for 15 minutes. The product is precipitated with 4 volumes of ethyl acetate and purified by HPLC.

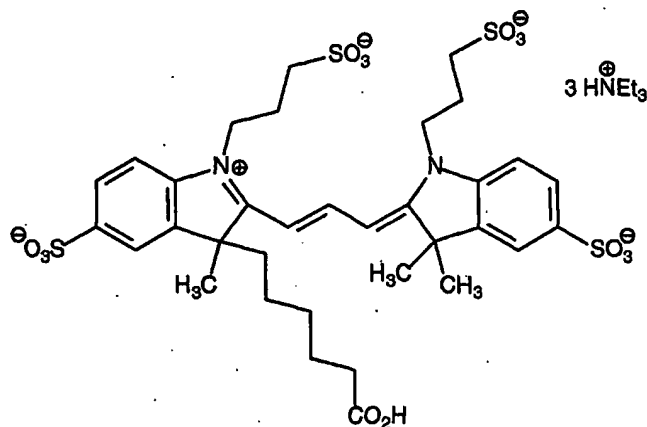
10

Example 11. Preparation of Compound 11.**Compound 11**

To Compound 9 in acetonitrile at room temperature is added 4 equivalents of triethylamine and 1.2 equivalents of N-(2-aminoethyl)maleimide, trifluoroacetic acid salt. The mixture is stirred at ambient temperature for 15 minutes. The product is precipitated with 4 volumes of ethyl acetate and purified by HPLC.

5

Example 12. Preparation of Compound 12.

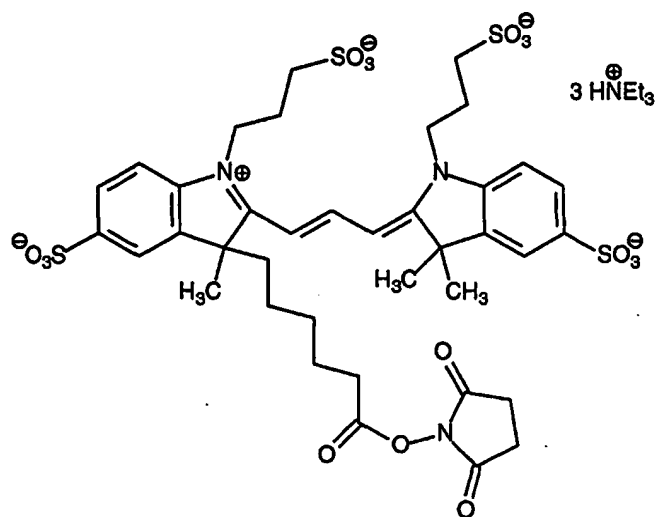


Compound 12

10 To 6 mmole of Compound 2 is added 2 g of Compound 5 in 20 mL DMF, 4.2 mL triethylamine, and 1.8 mL of acetic anhydride. The reaction is stirred at room temperature for one hour, then evaporated and the residue is purified by HPLC.

Example 13. Preparation of Compound 13.

15

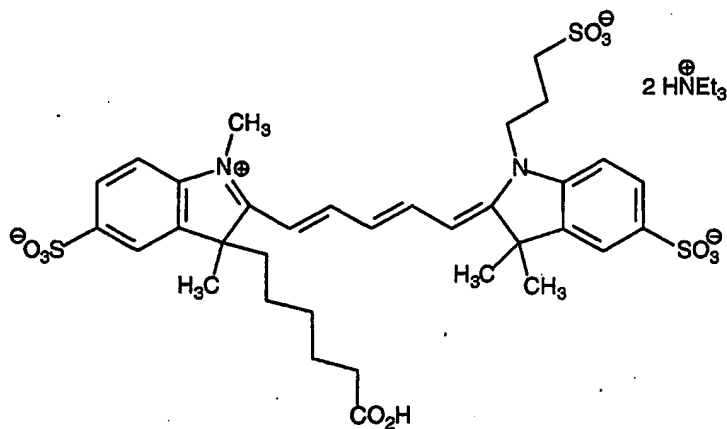


Compound 13

The procedure is similar to that used to prepare Compound 9, using Compound 12 in place of Compound 8.

5

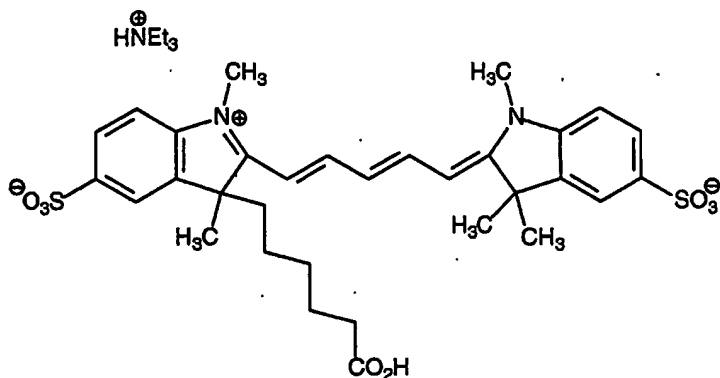
Example 14. Preparation of Compound 14.



Compound 14

10

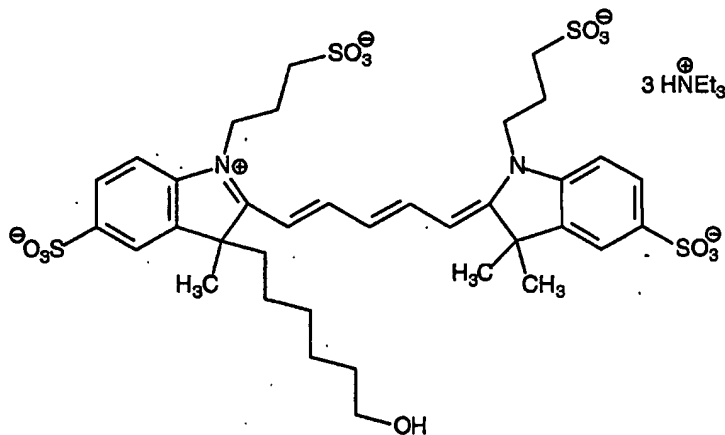
The compound is prepared in DMF by mixing one equivalent each of Compound 4 and Compound 7, followed by addition of four equivalents of triethylamine and 1.5 equivalents of acetic anhydride. After stirring at room temperature for 2 hours and evaporation, the residue is purified by HPLC.

Example 15. Preparation of Compound 15.**Compound 15**

- 5 To a mixture of 0.27 g Compound 6 and 0.6 mmoles Compound 7 in 8 mL of DMF is added 0.42 mL triethylamine and 0.1 mL acetic anhydride. The mixture is stirred at room temperature for two hours then evaporated and the residue is purified by HPLC.

Example 16. Preparation of Compound 16.

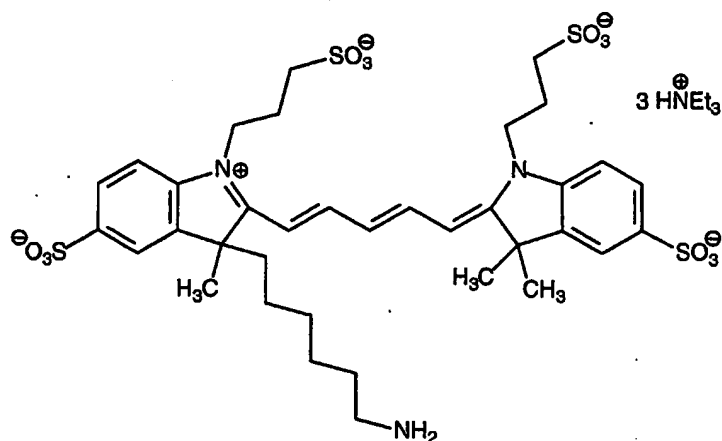
10

**Compound 16**

- 15 Ethyl 2-methylacetoacetate is alkylated with 6-benzoyloxy-1-bromohexane in the presence of 1.2 equivalents of sodium hydride in THF and the resulting product was hydrolyzed and decarboxylated in aqueous NaOH as in example 1 to generate the desired 9-hydroxy-3-methyl-2-nonanone. The nonanone is then heated at reflux with 1 equivalent of 4-hydrazinebenzenesulfonic acid in acetic acid to generate 3-(6-hydroxyhexyl)-2,3-

dimethyl-5-sulfoindolium, inner salt. The hydroxy again is protected as an benzoyloxy group and this intermediate is then transformed to the protected form of target compound as in example 8. The benzoyl protecting group is then removed by dilute NaOH.

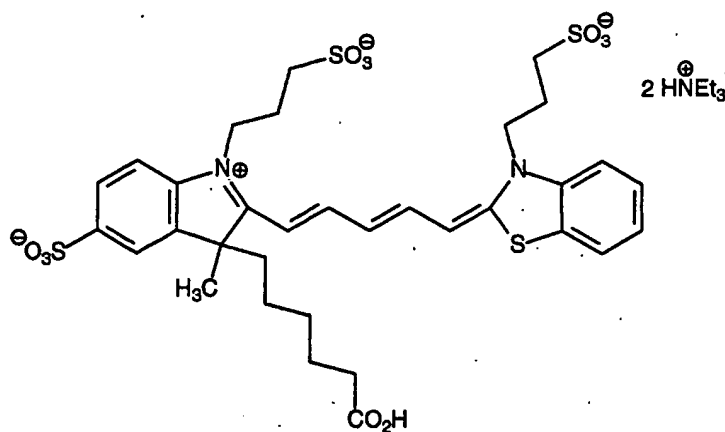
5 **Example 17. Preparation of Compound 17.**



Compound 17

10 The intermediate is prepared as in Example 16, except that 6-*t*-butoxycarbonyloxy-1-bromohexane is used instead of 6-benzoyloxy-1-bromohexane. The *t*-BOC protecting group is removed with trifluoroacetic acid at room temperature after formation of the target dye.

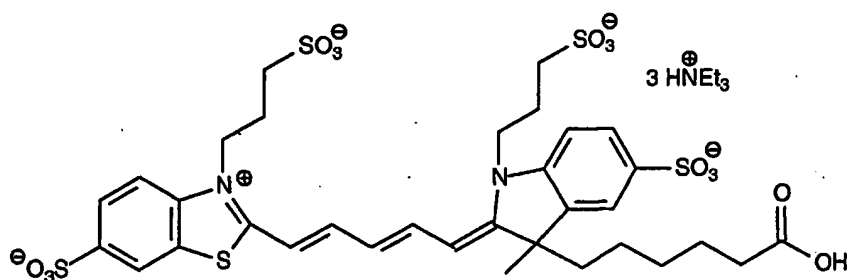
15 **Example 18. Preparation of Compound 18.**



Compound 18

To 1 mmole 2-methyl-1-(3-sulfopropyl)-benzothiazolium, inner salt (from heating of one equivalent each of propanesultone and 2-methylbenzothiazole at 110°C for one hour) and 150 mg of Compound 31 in 5 mL of DMF is added 0.28 mL of triethylamine and 0.1 mL acetic anhydride. The reaction is stirred at room temperature for one hour, then evaporated and the residue is purified by HPLC.

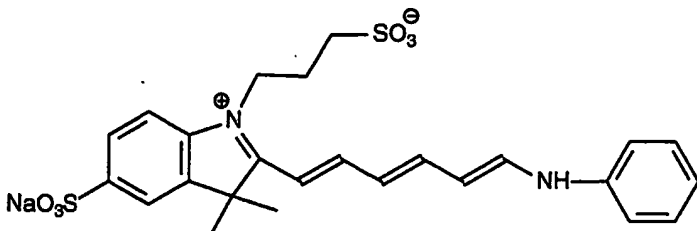
Example 19. Preparation of Compound 19.



Compound 19

Compound 19 is prepared in the same manner as Compound 18 except starting with 2-methyl-6-sulfobenzothiazole, which is prepared by reaction of sulfuric acid and 2-methylbenzothiazole at room temperature.

Example 20. Synthesis of 2-(6-anilinohexatrienyl)-3,3-dimethyl-5-sulfo-1-(3-sulfopropylindolium), inner salt (Compound 20).

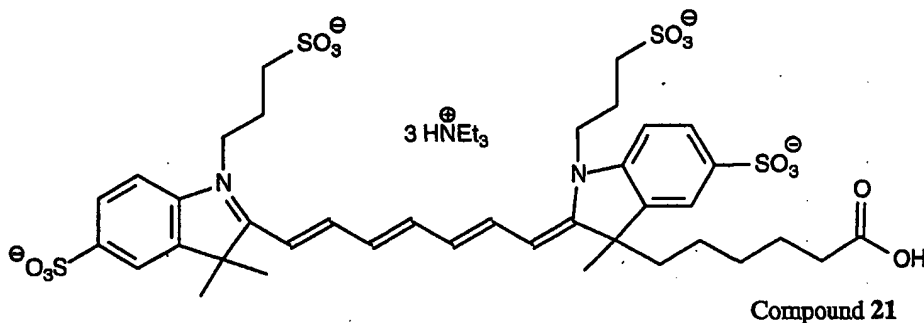


Compound 20

A mixture of 1.9 g of Compound 3A and 2.85 g of N-(5-anilino-2,4-pentadienylidene)aniline hydrochloride in 30 mL of acetic anhydride is heated at 120°C for 30 minutes. At the end of the period, 90 mL of ethyl acetate is added and the product is filtered and used as is.

5

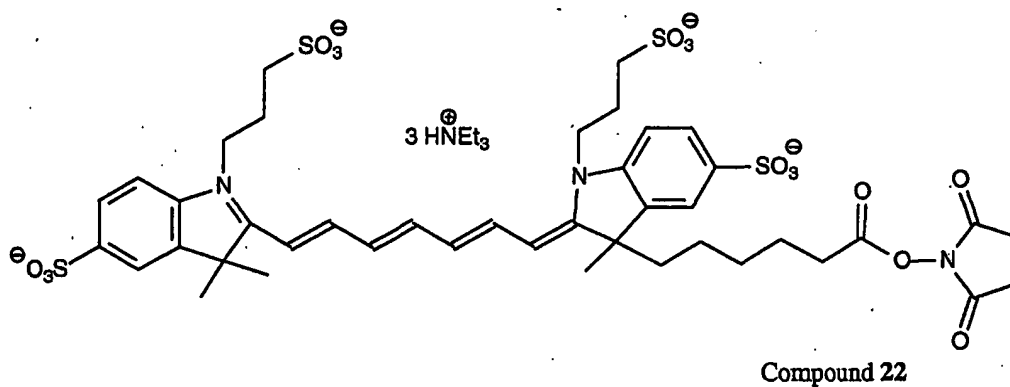
Example 21. Synthesis of Compound 21.



A mixture of 1.05 g of Compound 20, 2 mmoles of Compound 2, 10 mL of DMF, 1.7 mL of triethylamine and 0.6 mL of acetic anhydride is stirred at room temperature overnight and then at 35°C for an additional 1.5 hour. 40 mL of ethyl acetate is added and the precipitate is purified by HPLC.

10

Example 22. Synthesis of Compound 22.

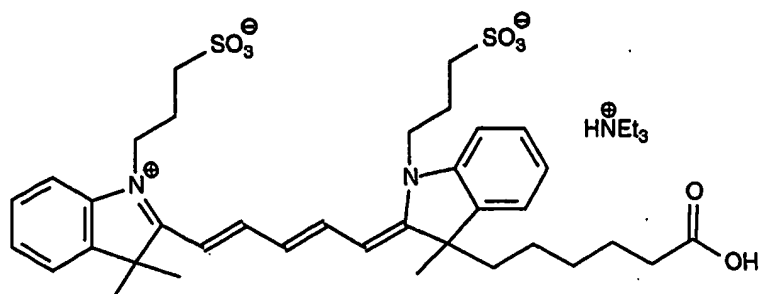


15

The succinimidyl ester of Compound 21 (Compound 22) is prepared as described in Example 9.

Example 23. Synthesis of Compound 23.

20



Compound 23

A mixture of 0.37 g of 2-(4-anilinobutadienyl)-3,3-dimethyl-1-(3-sulfopropyl)indolium (prepared by the reaction of trimethylindoline and propanesultone as in Example 3 followed by reaction with malonaldehyde dianil hydrochloride as in

5 Example 4), 1.35 mmoles of 3-(5-carboxypentyl)-3-methyl-1-(3-sulfopropyl)indolium (prepared by the reaction of 7-methyl-8-oxononanoic acid and phenyl hydrazine, as in Example 1), 7 mL DMF, 0.42 mL triethylamine and 0.1 mL of acetic anhydride is stirred at room temperature for one hour. Ethyl acetate (30 mL) is added and the precipitate is purified on silica gel to yield 55 mg of Compound 23.

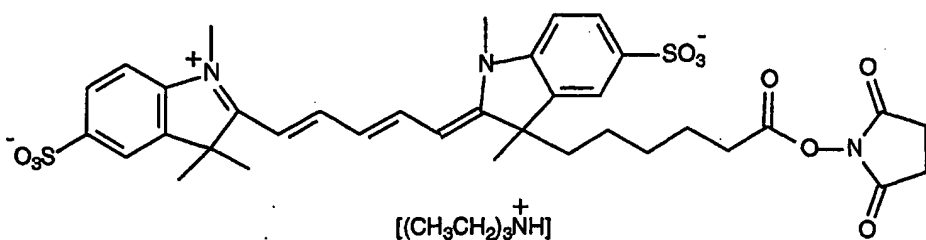
10

Example 24. Synthesis of Corresponding Activated Esters from Free Acids.

The following activated esters are prepared from the corresponding free acids, according to the method in Example 9:

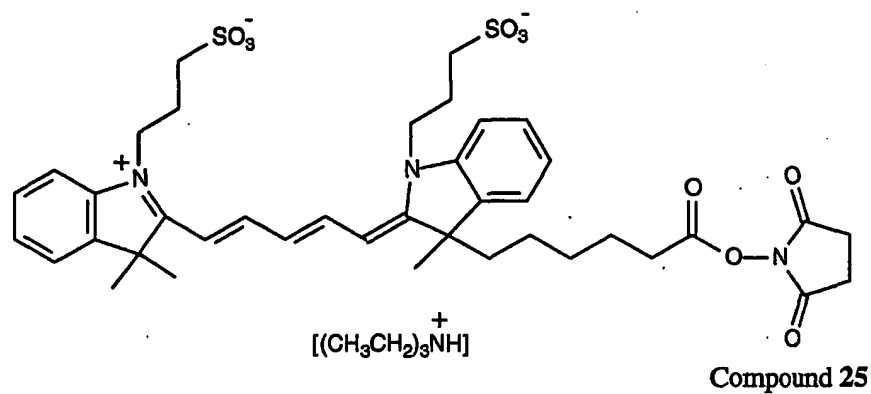
15

Compound 24, prepared from Compound 15

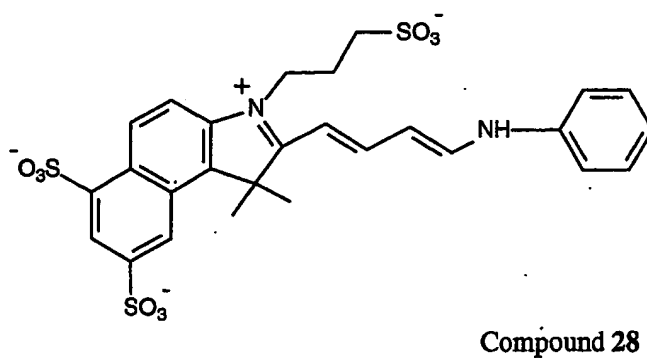


Compound 24

Compound 25, prepared from Compound 23

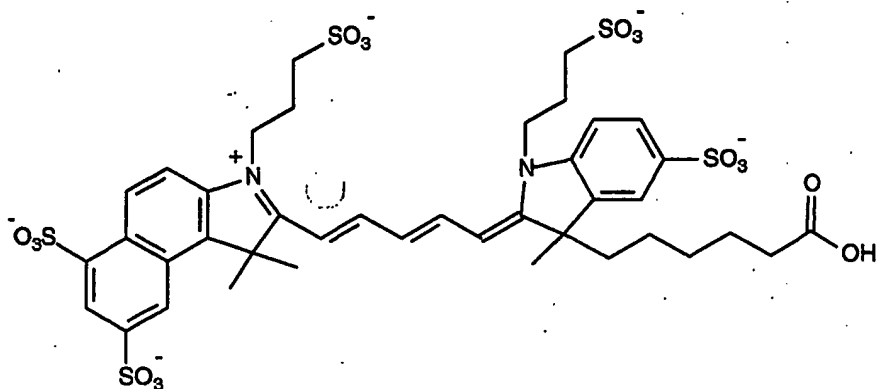


5 **Example 25. Preparation of Compound 28.**



- The compound is prepared by quaternization of 1,1,2
- 10 trimethylbenzindoleninium 1,3-disulfonate (Bioconjugate Chem., 356-362 (1996)) with propanesultone and then heated with 2 equivalents of malonaldehyde dianil hydrochloride in acetic acid with catalytic amount of triethylamine to yield Compound 28.

15 **Example 26. Preparation of Compound 29.**

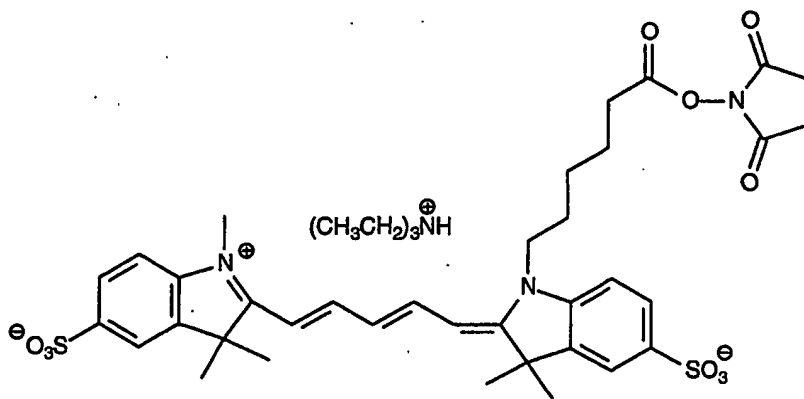


Compound 29

The compound is prepared by stirring one equivalent each of Compound 28 and 3-(5-carboxypentyl)-2,3-dimethyl-5-sulfo-1-(3-sulfopropyl)-indoleninium inner salt, sodium salt in the presence of 3 equivalents of triethylamine and one equivalent of acetic anhydride in DMF at room temperature for one hour to yield Compound 29. Compound 29 is optionally converted to its corresponding succinimidyl ester as described in Examples 9 and 24.

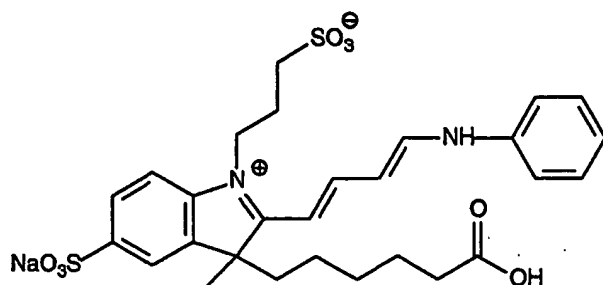
10 **Example 27. Preparation of Compound 30:**

For purposes of comparison with dyes of the invention (See Figure 1), Compound 30 is prepared according to BIOCONJUGATE CHEM. 4, 105-111 (1993).



Compound 30

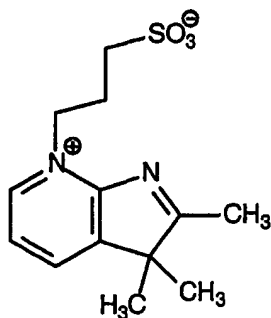
Example 28. Preparation of Compound 31.



Compound 31

A mixture of 45 mmoles of Compound 2 and 23 g of malonaldehyde dianil hydrochloride is heated to reflux in 400 mL of acetic acid with 0.65 mL of triethylamine for 1 hour. The solvent is evaporated and the residue is purified on silica gel to yield 2.4 g of the product.

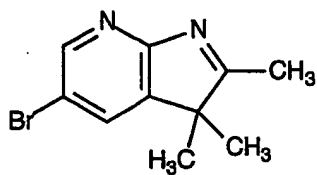
Example 29. Preparation of 7-(3-sulfopropyl)-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridinium, inner salt (Compound 51).



Compound 51

A mixture of 9 g of 2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridine and 20.6 g of propanesultone is heated at 60 °C for 3 hours. The reaction mixture is then dissolved in 100 mL of acetonitrile and 300 mL of ethyl acetate is added. The resulting sticky solid is again stirred in 300 mL of ethyl acetate to yield 22 g of the product.

Example 30. Preparation of 5-bromo-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridine (Compound 52).



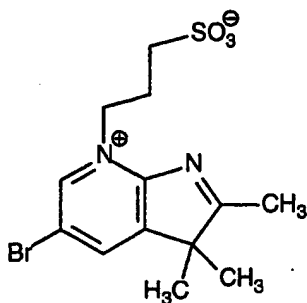
Compound 52

5

To 40 g of 2,5-dibromopyridine in 200 mL of 2-methoxyethanol is added 53 mL of anhydrous hydrazine and the mixture is heated at 110 °C for 3 hours to generate the 5-bromo-2-hydrazinopyridine. A mixture of 10 g of this hydrazinopyridine is heated at reflux overnight with 11 mL of 3-methyl-2-butanone in 40 mL of benzene equipped, using a condenser equipped with a Dean-Stark trap. All of the volatile components are removed under reduced pressure and the resulting residue is heated in 62 g of polyphosphoric acid at 140 °C for 45 minutes. The reaction mixture is poured into water, neutralized with sodium hydroxide and extracted with ethyl acetate. The resulting crude residue is purified by chromatography on silica gel, eluting with 1:1 ethyl acetate/hexanes, to yield 1.44 g of the product.

10
15

Example 31. Preparation of 5-bromo-7-(3-sulfopropyl)-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridinium, inner salt (Compound 53).



20

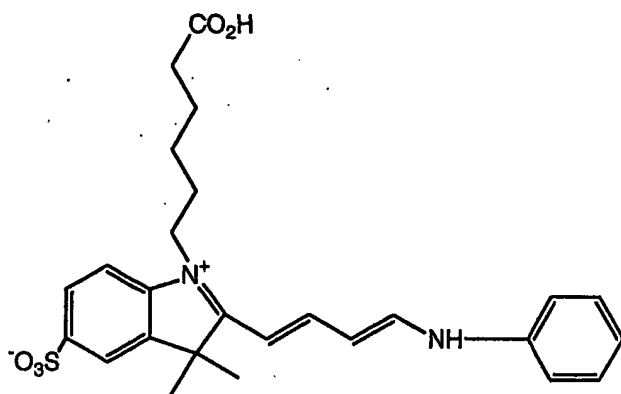
Compound 53

A mixture of 1 g of 5-bromo-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridine (Compound 52, Example 30) and 1.54 g of propanesultone is heated at 65 °C for 2 hours.

Ethyl acetate is added and the resulting mixture is stirred at room temperature overnight to yield 2.26 g of the product.

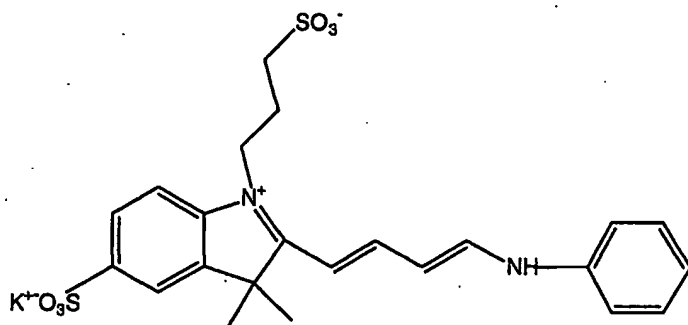
Example 32. Preparation of Compounds 54 and 55.

5



Compound 54

10



Compound 55

15

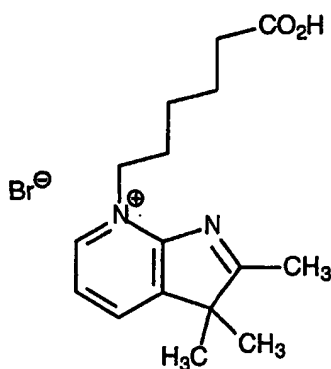
2-(4-Anilinobutadienyl)-1-(5-carboxypentyl)-3,3-dimethyl-5-sulfoindolinium, inner salt and 2-(4-anilinobutadienyl)-1-(3-sulfopropyl)-3,3-dimethyl-5-sulfoindolinium, potassium salt (Compounds 54 and 55, respectively) are prepared according to a literature procedure (Mujumdar, et al, BIOCONJUGATE CHEMISTRY 4, 105-111 (1993))

20

Example 33. Preparation of succinimidyl esters of carboxylic acids.

- Succinimidyl ester derivatives are typically prepared from the corresponding carboxylic acids using the 2-succinimido-1,1,3,3-tetramethyl uronium tetrafluoroborate (Bannwarth et al. TETRAHEDRON LETT. 1157-1160 (1991)) and either triethylamine or diisopropylethylamine. Succinimidyl ester derivatives are also readily prepared by coupling a carboxylic acid derivative to N-hydroxysuccinimide using an activating agent such as a carbodiimide.

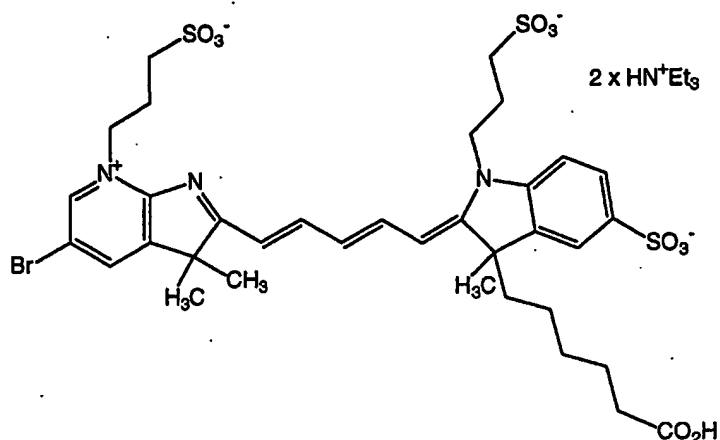
- Example 34. Preparation of 7-(carboxypentyl)-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridinium bromide (Compound 59).



Compound 59

- A mixture of 0.54 g of 2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridine and 1.32 g of 6-bromohexanoic acid is heated at 120 °C for one hour. Ethyl acetate (10 mL) is added, and the reaction mixture is heated at reflux for 15 minutes, then cooled to room temperature. The supernatant liquid is decanted to yield the product.

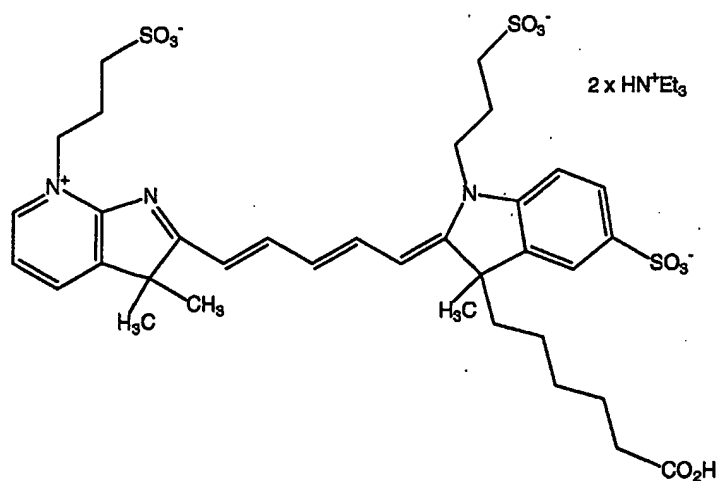
- Example 35. Preparation of Compound 66 and Compound 67.



Compound 66

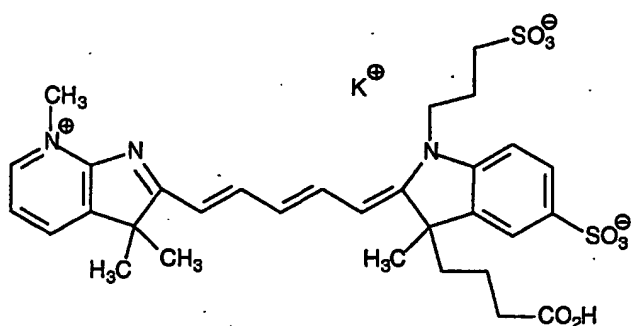
- 5 A mixture of 0.32 g of 2-(4-anilino-1,3-butadienyl)-3-methyl-5-sulfo-1-sulfopropylindolinium, sodium salt (Compound 31, Example 28), 0.75 g of 5-bromo-7-(3-sulfopropyl)-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridinium, inner salt (Compound 53, Example 10), 0.36 mL of triethylamine and 0.1 mL of acetic anhydride is stirred in 13 mL of DMF at room temperature for 1 h. 50 mL of ethyl acetate is added then the crude solid
- 10 is filtered and purified by HPLC. A succinimidyl ester derivative (Compound 67) is prepared according to the methods provided in Example 33.

Example 36. Preparation of Compound 68 and Compound 69.



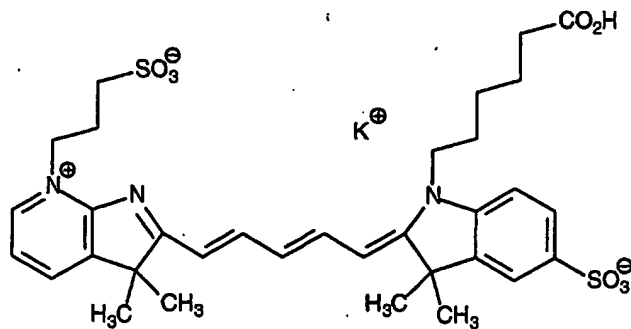
Compound 68

- The compound is prepared in a similar manner as Compound 66 except that 7-(3-sulfopropyl)-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridinium, inner salt (Compound 51, Example 8) is used. The crude product is purified by HPLC. A succinimidyl ester derivative (Compound 69) is prepared according to the methods provided in Example 33.

Example 37. Preparation of Compound 72.

Compound 72

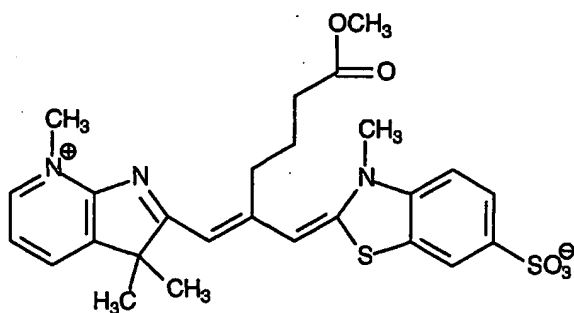
- A mixture of 150 mg of 2-(anilinobutadienyl)-3-(3-carboxypropyl)-5-sulfo-1-sulfopropylindolenium, inner salt, potassium salt, 160 mg of 2,3,3,7-tetramethyl-3H-pyrrolo[2,3-b]pyridinium tosylate, 2 mL of DMF, 0.13 mL of triethylamine and 0.08 mL of acetic anhydride is heated at 40 °C for 30 minutes. Volatile components are evaporated and the crude residue is purified on a silica gel column.

Example 38. Preparation of Compound 73 and Compound 74.

Compound 73

A solution of 3.2 g of 7-(3-sulfopropyl)-2,3,3-trimethyl-3H-pyrrolo[2,3-b]pyridinium, inner salt and 4 g of 2-(4-anilinobutadienyl)-1-(5-carboxypentyl)-3,3-dimethyl-5-sulfoindolinium, inner salt in 40 mL of DMF, 3.4 mL of triethylamine and 2 mL of acetic anhydride is stirred at room temperature for 4 hours. At the end of the period, 700 mL of acetonitrile is added and the crude solid is recovered by filtration and purified by HPLC. The succinimidyl ester derivative (Compound 74) is generated by the methods provided in Example 33.

10 Example 39. Preparation of Compound 79.

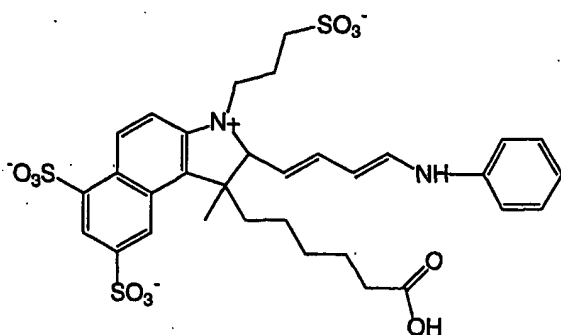


Compound 79

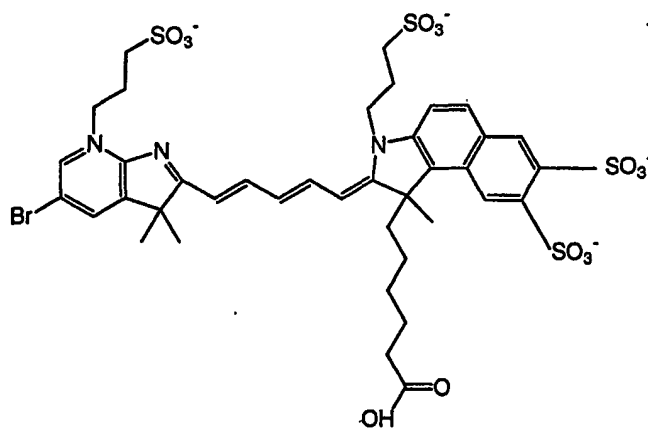
To 1.7 g of 2,3-dimethyl-6-sulfobenzothiazolium tosylate in 20 mL of pyridine at room temperature is added 1.03 mL of methyl 5-chloro-5-oxovalerate. The mixture is heated at 50-60 °C for 3 hours. The pyridine solvent is removed under reduced pressure, and the reaction is worked up with chloroform and brine, and purified by silica gel column to yield 0.92 g of 2-(5-methoxycarbonyl-2-oxopentylidene)-3-methyl-3H-benzothiazole. A mixture of 0.45 g of this benzothiazole and 0.45 g of phosphorous oxychloride in 5 mL of dichloroethane is heated at reflux for 2 hours to generate 2-(2-chloro-2-methoxycarbonylpropylvinyl)-3-methylbenzothiazolium chloride. The volatile components are evaporated and the crude chloride is used without further purification. The crude chloride and 0.45 g of 2,3,3,7-tetramethyl-3H-pyrrolo[2,3-b]pyridinium tosylate is stirred in 5 mL of dichloroethane in the presence of 0.45 mL of triethylamine for 2 hours. The volatile components are removed under reduced pressure, and the residue is dissolved in 5 mL of methanol and added dropwise to a solution of 4.5 g of sodium iodide in 30 mL water. The sticky solid is purified on a silica gel column to yield Compound 79.

Example 40. Preparation of Compound 82 and Compound 86.

6-Hydrazinonaphthalene 1,3-disulfonate (Bioconj. Chem., 356-362(1996)) is heated with
5 7-methyl-8-oxononanoic acid in acetic acid to generate the 1-carboxypentyl-1,2-dimethyl-
6,8-disulfobenzindolenine. The benzindolenine is quarternized with propane sultone and
chain elongated with malonaldehyde dianil hydrochloride to yield the 2-(4-
anilinobutadienyl) derivative (Compound 82) which is then reacted with Compound 53
(Example 31) in the presence of acetic anhydride and triethylamine to yield the desired
10 product.



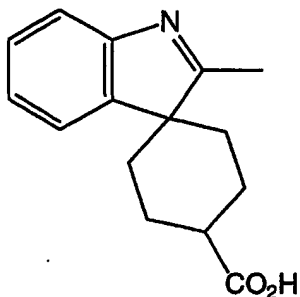
Compound 82



Compound 86

Example 41. Preparation of Compound 83.

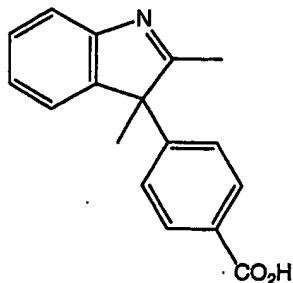
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**Compound 83**

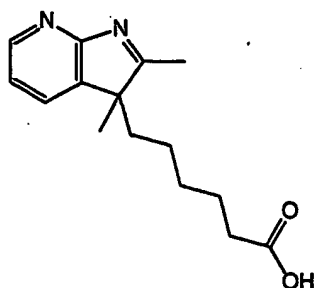
- 10 A mixture of 100 mg of 4-acetylcyclohexanecarboxylic acid and 120 mg of hydrazinobenzenesulfonic acid is refluxed in 5 mL of acetic acid to obtain the desired compound.

Example 42. Preparation of Compound 84.

15

**Compound 84**

- 20 A mixture of 2-(4-carboxyphenyl)-butan-2-one and hydrazinobenzenesulfonic acid in acetic acid is refluxed for 3 hours to obtain the desired product.

Example 43. Preparation of Compound 85.**Compound 85**

- 5 A mixture of 0.85 g of 2-hydrazinopyridine and 1.6 g of 7-methyl-8-oxo-nonanoic acid is refluxed in 10 mL of benzene overnight. The volatile components are evaporated and the residue is heated with 0.2 g of zinc chloride at 250° C for one hour to obtain the desired product.

10 **Example 44. Preparation of protein dye-conjugates.**

A series of dye conjugates of goat anti-mouse IgG (GAM), goat anti-rabbit IgG (GAR), streptavidin, transferrin and other proteins, including R-phycoerythrin (R-PE) and allophycocyanin (APC) are prepared by standard means (Haugland et al., METH. MOL.

- 15 BIOL. 45, 205 (1995); Haugland, METH. MOL. BIOL. 45, 223 (1995); Haugland, METH. MOL. BIOL. 45, 235 (1995); Haugland, CURRENT PROTOCOLS IN CELL BIOLOGY 16.5.1-16.5.22 (2000)) using Compound 9 or 74 and a mono-succinimidyl ester derivative of the Cy5 dye (Amersham-Pharmacia Biotech).
- 20 The typical method for protein conjugation with succinimidyl esters of the invention is as follows. Variations in ratios of dye to protein, protein concentration, time, temperature, buffer composition and other variables that are well known in the art are possible that still yield useful conjugates. A solution of the protein is prepared at ~10 mg/mL in 0.1 M sodium bicarbonate. The labeling reagents are dissolved in a suitable solvent such as
- 25 DMF at ~10 mg/mL. Water is a suitable solvent for many dyes of the invention. Predetermined amounts of the labeling reagents are added to the protein solutions with stirring. A molar ratio of 10 equivalents of dye to 1 equivalent of protein is typical,

though the optimal amount varies with the particular labeling reagent, the protein being labeled and the protein's concentration, and is determined empirically.

When optimizing the fluorescence yield and determining the effect of degree of substitution (DOS) on this brightness, it is typical to vary the ratio of reactive dye to protein over a several-fold range. The reaction mixture is incubated at room temperature for one hour or on ice for several hours. The dye-protein conjugate is typically separated from free unreacted reagent by size-exclusion chromatography, such as on BIO-RAD P-30 resin equilibrated with phosphate-buffered saline (PBS). The initial, protein-containing colored band is collected and the degree of substitution is determined from the absorbance at the absorbance maximum of each fluorophore, using the extinction coefficient of the free fluorophore. The dye-protein conjugate thus obtained can be subfractionated to yield conjugates with higher, lower or more uniform DOS.

A solution of the desired protein is prepared at 10 mg/mL in 0.1 M sodium bicarbonate. The labeling reagents are dissolved in DMF at 10 mg/mL. Predetermined amounts of the labeling reagents are added to the protein solutions with stirring. A molar ratio of 10 equivalents of dye to 1 equivalent of protein is typical, though the optimal amount varies with the particular labeling reagent, the protein being labeled and the protein's concentration, and is determined empirically. The reaction mixture is incubated at room temperature for one hour, or on ice for several hours. The dye-protein conjugate is typically separated from free unreacted reagent by size-exclusion chromatography on BIO-RAD P-30 resin equilibrated with PBS. The initial, protein-containing colored band is collected and the degree of substitution is determined from the absorbance at the absorbance maximum of each fluorophore, using the extinction coefficient of the free fluorophore.

Table 5: Fluorescence of Protein Conjugates of Compound 69 (Example 36)

Protein	DOS*	Quantum Yield†
Goat anti-Mouse IgG	3.7	0.47
Streptavidin	4.5	0.85
Wheat Germ Agglutinin	3.1	0.32
Goat anti-Rabbit IgG (highly cross-absorbed)	4.4	0.5
Goat anti-Chicken IgG	4.5	0.33

Rabbit anti-Mouse IgG	3.0	0.67
Goat anti-Mouse IgG (highly cross-absorbed)	4.4	0.63
Goat anti-Guinea Pig IgG	5.1	0.33
Protein A (MR=4)‡	2.1	0.64
Protein A (MR=8)‡	4.9	0.39

*Extinction coefficients are determined for the free carboxylic acid in aqueous solution

†Quantum yield measured relative to DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridine-2-one)

‡MR is the approximate molar ratio of the dye to protein following conjugation

5

Table 6: Fluorescence of Protein Conjugates of Compound 67 (Example 35)

Protein	DOS*	Quantum Yield
Goat anti-Mouse IgG	5.9	0.15
Streptavidin	4.8	0.66
Goat anti-Rabbit IgG (highly cross-absorbed)	4.9	0.26
Goat anti-Chicken IgG	---	---
Rabbit anti-Mouse IgG	6.0	0.31

*Extinction coefficients are determined for the free carboxylic acid in aqueous solution

Protein conjugates of antibody fragments, of other avidins and of other proteins are prepared and analyzed similarly.

10

Example 45. Total fluorescence of selected dye-protein conjugates as a function of degree of substitution (DOS).

15 The conjugates of Compound 9 exhibit equal or greater fluorescence than the conjugates of Cy5 dye at similar DOS when conjugated to a wide variety of proteins (Table 3). Protein conjugates are prepared (as described in Example 44) at varying DOS and compared for brightness (total-fluorescence, TF) and relative quantum yield (RQY; definition at bottom of Table 3). Total fluorescence is proportional to the overall

20 brightness of the bioconjugate, and is defined as the product of the RQY and DOS: $TF = RQY \times DOS$.

Example 46. Total fluorescence of selected dye-protein conjugates as a function of degree of substitution.

Table 3 shows the heavy quenching of the fluorescence of Cy5 conjugates (at even moderate DOS). Comparing GAM IgG Compound 9 at ~ DOS 4.2 with a GAM IgG Cy5 at DOS ~ 4.8 (see Table 3), reveals that the Compound 9 bioconjugate is approximately 5.0/0.4 brighter (12.5X) than the Cy5 bioconjugate. This type of pattern is observed for all of the proteins in Table 3. In general, the higher the DOS, the brighter Compound 9 bioconjugates are relative to the Cy5 bioconjugates, although, as can be seen, the Compound 9 bioconjugates are brighter at all DOS tested.

The decrease in the RQY of the Cy5 bioconjugates is found to be accompanied by an increase in the 600-nm absorbance band relative to the 650-nm absorbance band. This effect is true for all of the bioconjugates listed in Table 3. The increase in extinction of the 600 nm band is always associated with a large quenching of the fluorescence. Gruber et. al. (supra) who observed a similar correlation of an increased absorbance at 600 nm and a large decrease in fluorescence intensity. This general observation has now been confirmed with several other proteins (Table 3).

The Cy5 and Compound 9 derivatives of GAM are examined using both absorbance spectroscopy and fluorescence excitation spectroscopy. The fluorescence excitation spectra (emission wavelength = 725 nm) of each derivative is normalized to its absorbance spectra at 660 nm. The Cy5 GAM 600 nm absorbance band does not emit fluorescence, as evidenced by the large difference between the excitation and absorbance in this region of the spectrum. The absorbance and excitation spectra of Compound 9 bioconjugates nearly overlap in this region and hence show a drastically reduced amount of fluorescence quenching.. When this same Cy5-GAM antibody is dissolved in 6.0 M guanidinium hydrochloride (pH = 7.5), the 600 nm absorbance band greatly decreases, the 650 nm absorbance band increases, and the overall fluorescence intensity increases dramatically. This result indicates that it is the behavior of the Cy5 derivative on the bioconjugate native structure, that causes the large decrease in fluorescence. A similar result was obtained for Cy5 derivatives of nuclease-digested DNA (Example 59).

Example 47. Comparison of two structural "Cy5-like" isomers (Compound 30 and Compound 24).

In order to better understand the origin of the anomalous absorption effects of Cy5
5 bioconjugates and the much smaller effect with Compound 9, two isomers of Cy5 were
synthesized and conjugated to GAR at various DOS (Compound 30 and Compound 24).
Figure 4 is a direct comparison of absorbance properties of these two isomers at DOS's of
approximately 2.8, 4.3, and 5.5 on GAR. The only difference between the chemical
structures of Compounds 30 and 24 is the change in position of the reactive moiety from
10 position 1 (Cy5 position) to position 3 (shared by all dyes of the invention) of the indolium
ring. One can see that this chemical change has resulted in a drastic improvement of the
behavior of the absorbance of Compound 24 over Compound 30. Compound 24 is also a
brighter fluorescence emission than Compound 30 at all of these tested DOS's (Table 3).

15 Example 48. Comparison of the total fluorescence of GAM conjugates

Conjugates of the Cy5 dye with GAM were purchased from several sources (Table 3).
Absorption spectra of each of these conjugates confirm that the Cy5 GAM conjugates all
had much larger absorbances at 600 nm (relative to the 650 nm band) than the
20 corresponding Compound 9-GAM conjugates. For fluorescence brightness comparisons,
the proteins are adjusted to approximately the same concentration as measured by the
absorption at 280 nm corrected for the contribution of dye absorption at 280 nm. For
some commercial Cy5 bioconjugates, the protein concentration supplied by the vendor
was utilized due to the presence of 280 nm absorbing stabilizers. The conjugates are
25 excited at 633 nm and the fluorescence emission spectrum measured. The results in Table
3 confirm the superior fluorescence brightness (TF) of GAM conjugates of Compound 9
compared with the commercially available Cy5 conjugates of GAM.

The increased brightness is also apparent in experiments performed on a flow cytometer.
30 The flow cytometer intensity comparisons do not rely on the same type of RQY, DOS, and
TF calculations required for the spectroscopically based fluorescence intensity
comparisons.

Human peripheral blood is drawn in a sodium heparin tube. One hundred μL of blood is added to a Falcon tube. The blood is blocked with 5 μL of mouse monoclonal antibodies to both human CD16 and CD32 (Caltag) for 15 minutes at room temperature. The cells are washed with PBS and resuspended to 100 μL . The blood is then incubated with mouse monoclonal antibody to mouse anti-CD3 (Caltag) at the recommended concentration of 0.50 μg for 30 minutes at 37 degrees Celsius. After incubation with the primary antibody, the cells are washed and resuspended. The blood is then incubated with GAM conjugates of Compound 9 (prepared as in Example 44) and the commercial GAM conjugates of Cy5 from Jackson Laboratories (DOS \sim 1.9) and Amersham-Pharmacia (DOS \sim 11) at a concentration of 0.50 μg for 30 minutes at 37 degrees Celsius. The red blood cells are lysed with a cell lysis buffer and centrifuged to remove the lysed red blood cells. The cell pellet is washed once with PBS and resuspended to a final volume of 500 μL . The samples are analyzed on a FACScan flow cytometer (BD Biosciences) exciting with a 488 nm argon-ion laser and a long-pass (>650 nm) filter. The geometric mean of the background subtracted fluorescence intensity for the Compound 9 conjugates of GAM is 164, whereas the Cy5-GAM conjugates prepared by Jackson Laboratories and Amersham-Pharmacia are 71, and 30, respectively.

Flow cytometry studies are performed as a function of DOS for this antibody, and it is found that at all DOS's, the conjugate of Compound 9 with GAM is from 1.4X to 5.9X brighter than the commercially available Cy5 conjugates of GAM (see Table 7).

Table 7		
DOS	(Compound 9 GAM) / (Amersham Cy5 GAM) [†]	(Compound 9 GAM) / (Jackson Cy5 GAM) [‡]
1.83	4.84	1.92
2.36	5.7	2.26
3.94	5.8	2.26
4.36	5.86	2.33
7.25	3.6	1.4

[†], [‡], The geometric mean of the background subtracted fluorescence intensities obtained from Compound 9-labeled GAM divided by the intensity of Amersham-Pharmacia Biotech ([†]) or the Jackson Laboratories ([‡]) Cy5 version of this same antibody.

Example 49. GAR conjugates of Compound 13 and those of the spectrally similar CY3 dye.

GAR is labeled with Compound 13 and the CY3 reactive dyes at a variety of degrees of substitution ranging from 1.0–12. In all cases, the GAR conjugates of Compound 13 are superior in brightness to the Cy3 dye-labeled GAR (at equivalent DOS). A typical
5 example is shown in Figure 2 (DOS ~6.3). Excitation wavelength = 532 nm.

Example 50. Photostability comparison.

Photobleaching experiments are performed in small capillary tubes at 0.5 μ M
10 concentrations of Compound 9 and commercially available Cy5 reactive succinimidyl esters, in PBS, pH 7.5. The 40X objective of a Nikon Eclipse E-400 and Cy3/Cy5 filter XF92 (Omega) with a 100W Mercury lamp excitation, is utilized. Integrated intensities are collected under constant illumination as a function of time (Figure 3). After 100 minutes of illumination, Compound 9 remains about 2X brighter than the Cy5 dye.

15

Example 51. Labeling β -galactosidase with a thiol-reactive dye.

A solution of β -galactosidase, a protein rich in free thiol groups, is prepared in PBS (2.0 mg in 400 μ L). The protein solution is then treated with a 20 mg/mL solution of the
20 maleimide derivative Compound 11 in DMF. Unreacted dye is removed on a spin column. The degree of substitution by the dye is estimated using the extinction coefficient of the free dye. The protein concentration is estimated from the absorbance at 280 nm, corrected for the absorbance of Compound 11 at that wavelength.

Example 52. Fluorescence energy transfer in conjugates of R-phycoerythrin and
25 Allophycocyanin.

R-phycoerythrin (R-PE) conjugates of Compound 9 and 74 are prepared as in Example 44 with a DOS sufficiently high to quench the donor fluorescence almost completely (DOS ~ 4–8). The resulting phycobiliprotein conjugate is excited at 488 nm and the fluorescence
30 emission is compared to that of unmodified R-phycoerythrin excited at the same wavelength. Highly efficient energy transfer (> 99%) occurs from the protein to the fluorescent dye.

Compound 22 conjugated to R-PE with a DOS of 4.7, 8.2, and 13, generates energy-transfer efficiencies of ~90%, ~99%, and ~99.3%, respectively. A conjugate of these complexes with streptavidin is prepared essentially as described by Haugland (METH. MOL. BIOL. 45, 205 (1995), *supra*). This streptavidin conjugate retains the energy transfer properties and is useful for cell staining in flow cytometers that utilize the argon-ion laser for excitation.

Tandem conjugates of allophycocyanin can also be made, with longer wavelength dyes of the invention such as Compound 22 yield emission well beyond 700 nm when excited near 633 nm.

Human peripheral blood is drawn in a sodium heparin tube as in Example 48, except that a biotinylated anti-CD3 antibody (Caltag) is utilized as the primary step, and tandem-dye conjugates of Compound 9-derivatized streptavidin-R-PE and the commercial Cy5 version of this product (Gibco Red 670) are utilized for detection in parallel experiments. The samples are analyzed on a FACScan flow cytometer (BD Biosciences) exciting with a 488 nm argon-ion laser and a long-pass (>650 nm) filter. The signal-to-noise ratio of the tandem conjugate prepared from Compound 9 is ~ 4.5X brighter than the corresponding Cy5 tandem conjugate.

Example 53. Preparation of fluorescent-dye labeled microspheres.

Uniform microspheres are conjugated to the dyes of the invention by one of four methods. In Method A, 1.0 μm amine-derivatized polystyrene microspheres are suspended at ~2% solids in 100 mM NaHCO_3 , pH 8.3 and treated with 2 mg/mL of an amine-reactive dye. After 1 hour the microspheres are centrifuged and washed with buffer.

In Method B, carboxylate-modified microspheres are suspended in a solution of a protein that has been conjugated to a dye of the invention. The protein is passively adsorbed on the microspheres, and excess protein is removed by centrifugation and washing. Microparticles of a size that cannot be centrifuged are separated from excess protein by dialysis through a semi-permeable membrane with a high MW cutoff or by gel filtration chromatography.

In Method C a dye-labeled protein is covalently coupled through its amine residues to the carboxylate groups of the polymer using ethyl 3-(dimethylaminopropyl)carbodiimide (EDAC).

5

In Method D, biotinylated microspheres are treated with a streptavidin, avidin or anti-biotin conjugate of a dye of the invention, and the conjugates are isolated as in Method B.

The larger particles can be analyzed for uniformity of staining and brightness using flow cytometry. The microspheres can be further coupled to proteins, oligonucleotides, haptens and other biomolecules for assays using methods well known in the art.

10

Example 54. Preparation of fluorescent liposomes using dyes of the invention.

Selected dyes of the invention are sufficiently water soluble to be incorporated into the interior of liposomes by methods well known in the art (J. BIOL. CHEM. 257, 13892 (1982) and PROC. NATL. ACAD. SCI. USA 75, 4194 (1978)). Alternatively, liposomes containing dyes of the invention having a lipophilic substituent (e.g. alkyl having 11-22 carbons), within their membranes are prepared by co-dissolving the fluorescent lipid and the unlabeled lipids phospholipid(s) that make up the liposome before forming the liposome dispersion essentially as described by Szoka, Jr. et al. (ANN. REV. BIOPHYS. BIOENG. 9, 467 (1980)).

20

Example 55. Preparation of nucleotide dye-conjugates.

25

To 2 mg of 5-(3-aminoallyl)-2'-deoxyuridine 5'-triphosphate (Sigma Chemical) in 100 μ L water is added Compound 9 or 69 in 100 μ L DMF and 5 μ L triethylamine. After 3 hours, the solution is evaporated and the residue is purified by HPLC. The product fractions are lyophilized to give the red-fluorescent nucleotide conjugate.

30

Alternatively, fluorescent dye-conjugates of deoxyuridine 5'-triphosphate are prepared from 5-(3-amino-1-propynyl)-2'-deoxyuridine 5'-triphosphate (as described in Hobbs, Jr.

et al, *supra*), or by treating a thiolated nucleotide or a thiophosphate nucleotide with a thiol-reactive dye of the invention (such as the maleimide Compound 11).

- 5 Additionally, 2'-(or 3')-2-aminoethylaminocarbonyl-adenosine 5'-triphosphate is reacted with a slight excess of Compound 9 and, following precipitation with ethanol, the ribose-modified product is purified by preparative HPLC.

Example 56. Preparation of an oligonucleotide dye-conjugate.

- 10 A 5'-amine-modified, 18-base M13 primer sequence (~100 µg) is dissolved in 4 µL H₂O. To this is added 250 µg of Compound 9 or 69 in 100 µL 0.1 M sodium borate, pH 8.5. After 16 hours, 10 µL of 5 M NaCl and 3 volumes of cold ethanol are added. The mixture is cooled to -20°C, centrifuged, the supernatant is decanted, the pellet is rinsed with ethanol and then dissolved in 100 µL H₂O. The labeled oligonucleotide is purified by
15 HPLC on a 300Å C8 reverse-phase column using a ramp gradient of 0.1 M triethylammonium acetate (pH ~7) and acetonitrile (5-95% over 30 min). The desired peak is collected and evaporated to give the fluorescent oligonucleotide.

Example 57. In situ hybridization of an RNA probe.

- 20 Mouse fibroblasts are fixed and prepared for mRNA *in situ* hybridization using standard procedures. A dye-labeled RNA probe is prepared by *in vitro* transcription of a plasmid containing the mouse actin structural gene cloned downstream of a phage T3 RNA polymerase promoter. Labeling reactions comprise combining 2 µL DNA template (1 µg
25 DNA), 1 µL each of 10 mM ATP, CTP and GTP, 0.75 µL 10 mM UTP, 2.5 µL 1 mM aminoallyl-labeled UTP (Example 55), 2 µL 10X transcription buffer (400 mM Tris, pH 8.0, 100 mM MgCl₂, 20 mM spermidine, 100 mM NaCl), 1 µL T3 RNA polymerase (40 units/µL), 1 µL 2 mg/mL BSA, and 8.75 µL water. Reactions are incubated at 37°C for two hours.

- 30 The DNA template is removed by treatment with 20 units DNase I for 15 minutes, at 37°C. The RNA transcript is purified by extraction with an equal volume of phenol:chloroform, 1:1, then by chromatography on SEPHADEX G50. Labeled RNA is

denatured for 5 minutes at 50°C, then hybridized to cellular preparations using standard procedures. The long-wavelength fluorescence of the labeled cells is detected by excitation through an optical filter optimized for Cy5-like dyes (Omega XF47). The spatially integrated fluorescence from the FISH target region (18 separate intensities) as a function of the number of dyes incorporated per base of probe for conjugates of Compound 9 and of Cy5 reveal that conjugates of Compound 9 are brighter than the corresponding Cy5 probe by greater than 50% (at ~13 bases per dye incorporation).

10 Example 58. Preparing DNA hybridization probes using fluorescent platinum dye (ULS)-Compounds.

A fluorescent platinum complex (ULS) is prepared from dyes of the invention and from Cy5 monosuccinimidyl ester by adapting the methods provided in U.S. Pat. 5,714,327 to Houthoff et al. (1998). For each labeling reaction, a microfuge tube containing 1 µg of pUC1.77 plasmid DNA containing a chromosome 1 human α-satellite probe (DNase treated to a fragment size between 500–1000 bp) in 5 mM Tris, pH 8, 1 mM EDTA, is heated for ~10 minutes at 95°C to fully denature the DNA. The DNA is cooled on ice. 1 µL of a 1 mg/mL solution of the prepared ULS complex is added, followed by the addition of 5 mM Tris, pH 8, 1 mM EDTA to bring the total volume to 25 µL. The samples are incubated 15 minutes at 80°C. The reactions are stopped on ice. The labeled DNA is purified on a Bio-Rad Micro Bio-Spin P-30 Tris Chromatography Column. The labeled DNA products are suitable for *in situ* hybridization experiments.

A series of Compound 9 ULS and Cy5 ULS DNA hybridization probes are examined with the number of dyes per base varying from 0 per 100 bases, to approximately 8 dyes per hundred bases. Similar to the behavior of the Cy5 bioconjugates of proteins (Examples 33, 34) and on aminoallyl-labeled DNA (Example 60), the fluorescently quenched 600 nm absorbance band greatly increases at larger numbers of Cy5 dyes per DNA base (equivalent to higher DOS in protein examples) (open circles, Figure 4), but does not increase with the Compound 9 ULS-labeled DNA (closed circles, Figure 4).

Example 59. Preparing DNA hybridization probes using amine-modified DNA and an amine-reactive dye of the invention.

- Nick translation is performed using pUC1.77 plasmid DNA containing a chromosome 1 human α -satellite probe. To a microcentrifuge tube is added, in the following order: 23.5 μ L H₂O, 5 μ L 10X Nick Translation buffer (0.5 M Tris-HCl, 50 mM MgCl₂, 0.5 mg/mL BSA, pH 7.8), 5 μ L 0.1 M DTT, 4 μ L d(GAC)TP mix (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP), 1 μ L 0.5 mM dTTP, 4 μ L 0.5 mM aminoallyl-dUTP, 1 μ L 1 μ g/ μ L template DNA, 5 μ L DNase I (1 μ g/mL, 2000 Kunitz units/mg), 1.5 μ L DNA polymerase I (10 U/ μ L). The tube is incubated 2 hours at 15°C, then brought to a final volume of 100 μ L with H₂O. The amine-modified DNA is purified using a QIAQUICK PCR purification Kit (Qiagen) with the following modifications to purify the DNA from the enzyme and amine-containing Compounds: 75% EtOH is substituted for the wash buffer, H₂O is substituted for the elution buffer, and elution is performed twice for 5 minutes each. The DNA is precipitated by adding 1/10 volume 3 M sodium acetate and 2.5 volumes 100% EtOH, incubated at -70°C for 30 minutes, centrifuged for 15 minutes, and washed with 70% EtOH.

- The amine-modified DNA is resuspended in 5 μ L H₂O. To the solution is added 3 μ L 25 mg/mL sodium bicarbonate and 50 μ g of Compound 9 or 69 in 5 μ L DMF. The reaction is incubated for 1 hour at room temperature in the dark, to the reaction is added 90 μ L H₂O, and it is purified using a QIAQUICK PCR purification kit (QIAGEN), with the following modifications: three washes are performed with 75% EtOH and three elutions of 5 minutes each with the QIAGEN elution buffer. The DNA is precipitated as before. The labeled DNA products are suitable for *in situ* hybridization experiments, use on microarrays and as fluorescence donors or acceptors in hybridization-based assays. A comparison of varying the amino-allyl dUTP to dTTP ratios, followed by subsequent conjugation of the cDNA with either Compound 9 or Cy5 is shown in the Table 8. In the presence of excess reactive dye, both Compound 9 and Cy5 derivatives can label cDNA to equivalent degrees.

Table 8		
Nucleotide ratio, AA-dUTP:dTTP	Compound 9	Cy5
	bases / dye	bases / dye
90 μ M:10 μ M	13.2	13.6
60 μ M:10 μ M	14.8	16.1
30 μ M:10 μ M	17.6	15

10 μ M:10 μ M	18.1	18.9
3 μ M:10 μ M	23.3	23.7
1 μ M:10 μ M	30.2	32.2

Example 60. Comparison of the absorbance and fluorescence characteristics of nucleic acids.

5

Nucleic acid conjugates of Compound 9 and of Cy5 monosuccinimidyl ester are prepared from the same batch of amine-substituted nucleic acid (Example 59). Using the above protocol, it is possible to select a dye-labeling ratio that is essentially identical for both the Cy5 derivative and the Compound 9 derivative (e.g., 1 dye per 23 to 24 bases, Table 5).

- 10 Absorption spectra at the same nucleic acid concentration and dye labeling ratio show a shifting of extinction from the long-wavelength 650 nm band to a non-emitting 600 nm band for the Cy5 conjugate relative to the conjugate of Compound 9 (Figure 5). This result is very similar to the absorbance changes observed for Cy5 conjugates of proteins. Fluorescence emission spectra excited at 600 nm reveal ~4X greater fluorescence of the conjugate of Compound 9 cDNA relative to the Cy5 cDNA conjugate (Figure 5).
- 15

- The synthesized Cy5 labeled cDNA is digested with micrococcal nuclease (EC 3.1.31.1; Worthington Biochemicals). The enzyme is dissolved at 1.0 mg/mL in reagent-grade water and diluted to approximately 0.001 mg/mL in 0.1% bovine serum albumin prior to
- 20 addition to the Cy5 dye- and Compound 9-labeled cDNA's, pH = 8.8, 0.1 M sodium borate, 0.01 M calcium chloride. The reaction is allowed to proceed at room temperature for 4 hours.

- A comparison of the absorbance spectra of the identical labeled cDNA before and after
- 25 treatment with the nuclease (which will digest the cDNA to dNMP's) shows that the distortion in the absorbance of the Cy5-cDNA conjugate can be eliminated by digesting the cDNA down to its individual deoxynucleoside monophosphates. There is a concomitant increase in the fluorescence of the Cy5 cDNA upon digestion of ~15X, revealing that this absorbance pattern change is associated with the large decrease in the
- 30 fluorescence associated with the Cy5-cDNA conjugate.

Example 61. Discrimination of live and dead cells using the dyes of the invention.

Selected dyes of the invention are highly polar and therefore relatively impermeant to the membranes of live cells. These dyes can therefore be used to discriminate cells that have intact versus compromised cell membranes in a single-color assay as follows: Mouse

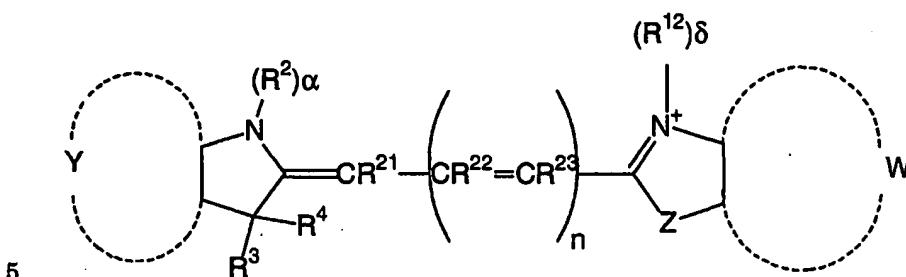
5 monocyte-macrophage, Abelson Leukemia Virus Transformed (RAW264.7) cells are trypsinized and washed with PBS, pH 7.2. Approximately 8–10 million cells suspended in 180 μ L of PBS, pH 7.2 are placed in a glass test tube and heated in a water bath at 50°C for 20 minutes to kill a fraction of the cells. Approximately 60 μ L (2–3 million cells) of the cell suspension is added to 940 μ L of PBS, pH 7.2, followed by 0.1 μ L of a 1 mg/mL

10 solution of a succinimidyl ester derivative of a dye of the invention in DMSO. The mixture is incubated on ice for 30 minutes and washed twice with PBS, followed by addition of 200 μ L of PBS, pH 7.2. An identical aliquot of cells is treated with 2 μ L of a 150 μ M solution of propidium iodide in water (as a control for dead cells). Analysis of the cell suspension using flow cytometry shows that populations of dead cells stained by the

15 instant Compounds and those stained by propidium iodide are very similar.

What is claimed is:

1. A compound of the formula:



and its salts, wherein

10 R^3 is $-L-R_x$; or $-L-S_y$; and R^4 is independently $-L-R_x$; or $-L-S_y$; or a C_1-C_{22} alkyl or C_7-C_{22} arylalkyl, each alkyl portion of which optionally incorporates up to six hetero atoms, selected from N, O and S, and each alkyl portion of which is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, C_1-C_6 alkoxy, C_1-C_6 alkylamino, or C_2-C_{11} dialkylamino, or C_2-C_{18} trialkylammonium; or R^3 and R^4 taken in combination
15 complete a five- or six-membered saturated or unsaturated ring that is substituted by $-L-R_x$; or $-L-S_y$; and

20 L is a single covalent bond, or a covalent linkage that is linear or branched, cyclic or heterocyclic, saturated or unsaturated, having 1-16 nonhydrogen atoms selected from the group consisting of C, N, P, O, and S, such that the linkage contains any combination of ether, thioether, amine, ester, amide bonds; or single, double, triple or aromatic carbon-carbon bonds; or phosphorus-oxygen, phosphorus-sulfur, nitrogen-nitrogen, nitrogen-oxygen, or nitrogen-platinum bonds; or aromatic or heteroaromatic bonds;

25 R_x is a reactive group;

S_y is a conjugated substance;

Y represents the atoms necessary to form one to two fused aromatic rings having 6 atoms in each ring, which atoms are selected from $-\text{CH}$, $-\text{C}$, $-\text{CR}^1$, and $-\text{N}(\text{R}^2)_\beta$, where each β is 0 or 1, and each R^1 is independently $-\text{L-R}_x$; or $-\text{L-S}_y$; or amino, sulfo, trifluoromethyl, or halogen; or $\text{C}_1\text{-C}_6$ alkyl, $\text{C}_1\text{-C}_6$ alkoxy, $\text{C}_1\text{-C}_6$ alkylamino, $\text{C}_2\text{-C}_{12}$ dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

each R^2 , when present (where α or β is 1), is independently $-\text{L-R}_x$; or $-\text{L-S}_y$; or a $\text{C}_1\text{-C}_{22}$ alkyl or $\text{C}_7\text{-C}_{22}$ arylalkyl, each alkyl portion of which optionally incorporates up to six hetero atoms, selected from N, O and S, and each alkyl portion of which is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, $\text{C}_1\text{-C}_6$ alkoxy, $\text{C}_1\text{-C}_6$ alkylamino, $\text{C}_2\text{-C}_{12}$ dialkylamino, or $\text{C}_8\text{-C}_{18}$ trialkylammonium;

15

α is 0 or 1, and $\alpha + \text{all } \beta = 1$; and

W represents the atoms necessary to form one to two fused aromatic rings having 6 atoms in each ring, which atoms are selected from $-\text{CH}$, $-\text{C}$, $-\text{CR}^{1'}$, and $-\text{N}(\text{R}^{2'})_\beta$, where each β' is 0 or 1, and each $\text{R}^{1'}$ is independently $-\text{L-R}_x$; or $-\text{L-S}_y$; or amino, sulfo, trifluoromethyl, or halogen; or $\text{C}_1\text{-C}_6$ alkyl, $\text{C}_1\text{-C}_6$ alkoxy, $\text{C}_1\text{-C}_6$ alkylamino, $\text{C}_2\text{-C}_{12}$ dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

each $\text{R}^{2'}$, when present (where δ or β' is 1), is independently $-\text{L-R}_x$; or $-\text{L-S}_y$; or is a $\text{C}_1\text{-C}_{22}$ alkyl or $\text{C}_7\text{-C}_{22}$ arylalkyl, each alkyl portion of which optionally incorporates up to six hetero atoms, selected from N, O and S, and each alkyl portion of which is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, $\text{C}_1\text{-C}_6$ alkoxy, $\text{C}_1\text{-C}_6$ alkylamino, $\text{C}_2\text{-C}_{12}$ dialkylamino, or $\text{C}_8\text{-C}_{18}$ trialkylammonium;

30

δ is 0 or 1, and $\delta + \text{all } \beta' = 1$;

Z is O, S, or $\text{CR}^{13}\text{R}^{14}$;

R^{13} and R^{14} are independently $-L-R_x$; or $-L-S_y$; or a C_1-C_{22} alkyl or C_7-C_{22} arylalkyl, each alkyl portion of which optionally incorporates up to six hetero atoms, selected from N, O and S, and each alkyl portion of which is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, C_1-C_6 alkoxy, C_1-C_6 alkylamino, or C_2-C_{12} dialkylamino, or C_8-C_{18} trialkylammonium; or R^{13} and R^{14} taken in combination complete a five- or six-membered saturated or unsaturated ring that is optionally substituted by $-L-R_x$; or $-L-S_y$;

10

n is 1, 2 or 3; and

each R^{21} , R^{22} , and R^{23} is independently H, F, Cl, C_1-C_6 alkyl, C_1-C_6 alkoxy, aryloxy, a nitrogen heterocycle, an iminium ion, $-L-R_x$, or $-L-S_y$; or any two adjacent substituents of R^{21} , R^{22} , R^{23} , when taken in combination, forms a 4-, 5-, or 6-membered saturated or unsaturated hydrocarbon ring that is optionally substituted one or more times by C_1-C_6 alkyl, halogen, or a carbonyl oxygen; or R^{21} taken in combination with R^4 forms a 6-membered ring that is optionally substituted by C_1-C_6 alkyl; or one of R^{23} , whichever is adjacent to Z, taken in combination with one of R^{13} and R^{14} forms a 6-membered ring that is optionally substituted by C_1-C_6 alkyl.

20

2. A compound, as claimed in Claim 1, wherein the atoms of Y are selected from $-CH$, $-C$, and $-CR^1$, where each R^1 is independently $-L-R_x$; or $-L-S_y$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

25

α is 1;

30 the atoms of W are selected from $-CH$, $-C$, $-CR^{1'}$, and $-N(R^{13})_{\beta'}$, where each β' is 0 or 1, and each $R^{1'}$ is independently $-L-R_x$; or $-L-S_y$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

each R^3 and R^{13} is independently $-L-R_x$; or $-L-S_x$; or a C_1-C_{22} alkyl, which optionally incorporates up to six hetero atoms, and is optionally substituted;

5 δ is 0 or 1, and $\delta + \text{all } \beta' = 1$; and δ is 1 only if W does not contain any ring nitrogen atoms;

Z is $CR^{13}R^{14}$.

10 3. A compound, as claimed in Claim 2, wherein the atoms of W are selected from $-CH$, $-C$, $-CR^{17}$, and $-N(R^{13})_{\beta'}$, where β' is 1, but no more than one of the atoms in W is $-N(R^{13})_{\beta'}$, and each R^{17} is independently $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy; and δ is 0.

15 4. A compound, as claimed in Claim 2, wherein the atoms of W are selected from $-CH$, $-C$, and $-CR^{17}$, and each R^{17} is independently $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

20 δ is 1.

5. A compound, as claimed in Claim 2, wherein R^3 is $-L-R_x$; or $-L-S_x$; and R^4 is independently a C_1-C_6 alkyl, which is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, or amino;

25

the atoms of Y are selected from $-CH$, $-C$, and $-CR^1$, where each R^1 is independently $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

30 the atoms of W are selected from $-CH$, $-C$, $-CR^{17}$, and $-N(R^{13})_{\beta'}$, where each β' is 0 or 1, but no more than one of such atoms is $-N(R^{13})_{\beta'}$, and each R^{17} is independently $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

- R^3 and R^{13} are independently $-L-R_x$; or $-L-S_x$; or is a C_1-C_6 alkyl, which is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, or amino;
- 5 one of R^{13} and R^{14} is a C_1-C_6 alkyl and the other of R^{13} and R^{14} is $-L-R_x$; or $-L-S_x$; or a C_1-C_{22} alkyl, which is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, C_1-C_6 alkoxy, C_1-C_6 alkylamino, or C_2-C_{12} dialkylamino;
- 10 each of R^{21} , R^{22} , and R^{23} are H; or any two adjacent substituents of R^{21} , R^{22} , R^{23} , when taken in combination, forms a 4-, 5-, or 6-membered saturated or unsaturated hydrocarbon ring that is optionally substituted one or more times by C_1-C_6 alkyl, halogen, or a carbonyl oxygen.
- 15 6. A compound, as claimed in Claim 1, wherein
- R^5 is $-L-R_x$; and R^4 is independently a C_1-C_{22} alkyl, which optionally incorporates up to six hetero atoms, and is optionally substituted; or R^5 and R^4 taken in combination complete a five- or six-membered saturated or unsaturated ring that is substituted by
- 20 $-L-R_x$; and
- each R_x is a reactive group that is independently an acrylamide, an activated ester of a carboxylic acid, an acyl azide, an acyl nitrile, an aldehyde, an alkyl halide, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a carboxylic acid, a diazoalkane, a haloacetamide, a halotriazine, a
- 25 hydrazine (including hydrazides), an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a sulfonyl halide, a thiol group, or a photoactivatable group;
- 30 no more than one of the atoms within Y is $-N(R^2)_2$, and each R^1 is independently $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy; and each S_x is a conjugated substance that is an amino acid, peptide, polypeptide, protein, nucleotide, oligonucleotide, nucleic acid

polymer, a sugar, a polysaccharide, an oligosaccharide, a fluorescent dye, or a microsphere;

α is only 1 when Y contains no ring nitrogen atoms,

5

no more than one of the atoms within W is $-N(R^{13})_p$, and each R^i is independently $-L-R_x$; or $-L-S_c$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

10

R^3 and R^{13} are independently $-L-R_x$; or $-L-S_c$; or is a C_1-C_{22} alkyl, which optionally incorporates up to six hetero atoms, and is optionally substituted;

δ is 1 only if W does not contain any ring nitrogen atoms;

15

Z is $CR^{13}R^{14}$;

R^{13} and R^{14} are independently $-L-R_x$; or $-L-S_c$; or a C_1-C_{22} alkyl, which optionally incorporates up to six hetero atoms, is optionally substituted;

20

or R^{13} and R^{14} taken in combination complete a five- or six-membered saturated or unsaturated ring that is optionally substituted by $-L-R_x$; or $-L-S_c$; and

R^{21} , R^{22} , and R^{23} are independently H, F, Cl, C_1-C_6 alkyl, C_1-C_6 alkoxy, aryloxy, a nitrogen heterocycle, an iminium ion, $-L-R_x$, or $-L-S_c$.

25

7. A compound, as claimed in Claim 1, wherein

R^3 is $-L-S_c$; and R^4 is independently a C_1-C_{22} alkyl, which optionally incorporates up to six hetero atoms, and is optionally substituted; or R^3 and R^4 taken in combination

30

complete a five- or six-membered saturated or unsaturated ring that is substituted by $-L-S_c$; and

L is a single covalent bond, or a covalent linkage and

- 5 S_i is a conjugated substance that is an antibody, an amino acid, an angiostatin or endostatin, an avidin or streptavidin, a biotin, a blood component protein, a dextran, an enzyme, an enzyme inhibitor, a hormone, an IgG-binding protein, a fluorescent protein, a growth factor, a lectin, a lipopolysaccharide, a metal-binding protein, a microorganism or portion thereof, a neuropeptide, a non-biological microparticle, a nucleotide, an oligonucleotide, a peptide toxin, a phospholipid-binding protein, a small-molecule drug, a structural protein, or a tyramide;
- 10 no more than one of the atoms within Y is $-N(R^2)_p$, and each R^1 is independently $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy; and $-R_x$ is a reactive group that is an activated ester of a carboxylic acid, an amine, a carboxylic acid, a halotriazine, a hydrazide, a
- 15 maleimide, a reactive platinum complex; and

α is only 1 when Y contains no ring nitrogen atoms,

- no more than one of the atoms within W is $-N(R^{13})_p$, and each R^1 is independently $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;
- 20

- R^2 and R^{12} are independently $-L-R_x$; or $-L-S_x$; or is a C_1-C_{22} alkyl, which optionally incorporates up to six hetero atoms, and is optionally substituted;
- 25

δ is 1 only if W does not contain any ring nitrogen atoms;

- 30 Z is $CR^{13}R^{14}$;

R^{13} and R^{14} are independently $-L-R_x$; or $-L-S_x$; or a C_1-C_{22} alkyl, which optionally incorporates up to six hetero atoms, is optionally substituted; or R^{13} and R^{14} taken in combination complete a five- or six-membered saturated or unsaturated ring that is optionally substituted by $-L-R_x$; or $-L-S_x$; and

R^{21} , R^{22} , and R^{23} , are independently H, F, Cl, C_1-C_6 alkyl, C_1-C_6 alkoxy, aryloxy, a nitrogen heterocycle, an iminium ion, $-L-R_x$, or $-L-S_c$.

5 8. A compound, as claimed in any of Claims 1-7, wherein

L is a covalent linkage that is linear or branched, cyclic or heterocyclic, saturated or unsaturated, having 1-16 nonhydrogen atoms selected from the group consisting of C, N, P, O, and S, and incorporating the formula $-(CH_2)_d(CONH(CH_2)_e)_z-$, where d is 0-5, e is 1-5 and z' is 0 or 1, such that the linkage contains any combination of ether, thioether, amine, ester, amide bonds; or single, double, triple or aromatic carbon-carbon bonds; or phosphorus-oxygen, phosphorus-sulfur, nitrogen-nitrogen, nitrogen-oxygen, or nitrogen-platinum bonds; or aromatic or heteroaromatic bonds.

15 9. A compound, as claimed in any of Claims 1-8, wherein, R^{21} , R^{22} , and R^{23} are H.

10. A compound, as claimed in any of Claims 1-9, wherein

S_c is a conjugated substance that is an actin, an antibody or fragment thereof, an avidin or streptavidin, a biotin, a dextran, an enzyme, a fluorescent protein, a lectin, a lipopolysaccharide, a microorganism, a non-biological microparticle, a nucleotide, an oligonucleotide, a peptide toxin, a phosphatidylserine-binding protein, a protein A or G, a small-molecule drug, or a tyramide.

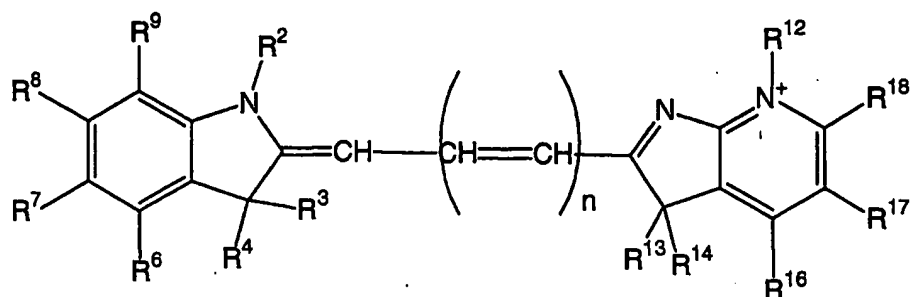
11. A compound, as claimed in any of Claims 1-10, wherein the atoms of Y are selected from $-CH$, $-C$, and $-CR^1$, where R^1 is sulfo.

12. A compound, as claimed in any of Claims 1-3 and 5-11, wherein the atoms of W are selected from $-CH$, $-C$, $-CR^1$, and $-N(R^{12})_3$, where each R^1 is sulfo or Br.

13. A compound, as claimed in any of Claims 1-12, wherein R^3 and R^{12} are independently a C_1-C_6 alkyl or C_1-C_6 alkyl substituted by sulfo.

14. A compound, as claimed in any of Claims 1-13, wherein, R^{12} and R^{14} are independently C_1-C_6 alkyl.

15. A compound of the formula



and its salts, wherein

- 5
 R^3 is $-L-R_x$; or $-L-S_y$; and R^4 is a C_1-C_{22} alkyl, and is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, C_1-C_6 alkoxy, C_1-C_6 alkylamino, or C_3-C_{12} dialkylamino, or C_3-C_{18} trialkylammonium; or R^3 and R^4 taken in combination complete a five- or six-
 10 membered saturated or unsaturated ring that is substituted by $-L-R_x$; or $-L-S_y$; and

- 15
 L is a single covalent bond, or a covalent linkage that is linear or branched, cyclic or heterocyclic, saturated or unsaturated, having 1-16 nonhydrogen atoms selected from the group consisting of C, N, P, O, and S, such that the linkage contains any combination of ether, thioether, amine, ester, amide bonds; or single, double, triple or aromatic carbon-carbon bonds; or phosphorus-oxygen, phosphorus-sulfur, nitrogen-nitrogen, nitrogen-oxygen, or nitrogen-platinum bonds; or aromatic or heteroaromatic bonds;

- 20
 R_x is a reactive group;

S_y is a conjugated substance;

- 25
 R^6 through R^9 and R^{12} through R^{18} are independently H, $-L-R_x$; or $-L-S_y$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_3-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy; or any two adjacent substituents of R^6 through R^9 form a fused benzo ring that is optionally substituted one or more times by $-L-R_x$; or by $-L-S_y$; or by amino, sulfo, trifluoromethyl, or halogen; or by C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino,

C₁-C₁₂ dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

R³ and R¹³ are independently a C₁-C₈ alkyl or C₁-C₈ alkyl substituted by sulfo;

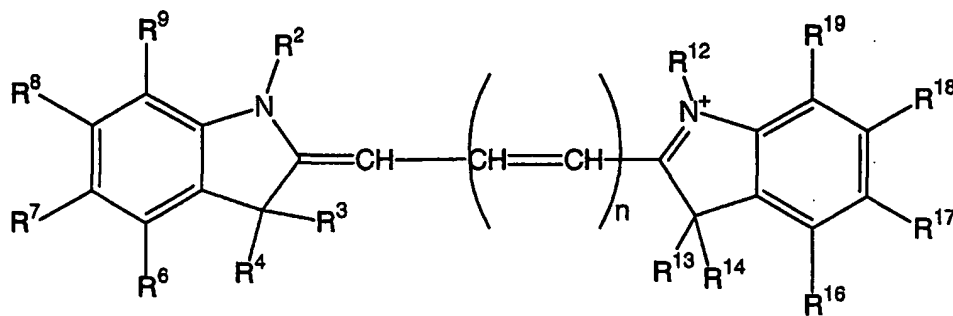
5

R¹³ and R¹⁴ are independently a C₁-C₈ alkyl;

n is 1, 2 or 3.

- 10 16. A compound, as claimed in Claim 15, wherein R¹⁷ is H or Br, and R¹⁸ and R¹⁹ are H.

17. A compound of the formula



5

and its salts, wherein

R^3 is $-L-R_x$; or $-L-S_x$; and R^4 is a C_1-C_{22} alkyl, and is optionally substituted one or more times by F, Cl, Br, I, hydroxy, carboxy, sulfo, phosphate, amino, sulfate, phosphonate, cyano, nitro, azido, C_1-C_6 alkoxy, C_1-C_6 alkylamino, or C_2-C_{12} dialkylamino, or C_3-C_{18} trialkylammonium; or R^8 and R^4 taken in combination complete a five- or six-membered saturated or unsaturated ring that is substituted by $-L-R_x$; or $-L-S_x$; and

L is a single covalent bond, or a covalent linkage that is linear or branched, cyclic or heterocyclic, saturated or unsaturated, having 1-16 nonhydrogen atoms selected from the group consisting of C, N, P, O, and S, such that the linkage contains any combination of ether, thioether, amine, ester, amide bonds; or single, double, triple or aromatic carbon-carbon bonds; or phosphorus-oxygen, phosphorus-sulfur, nitrogen-nitrogen, nitrogen-oxygen, or nitrogen-platinum bonds; or aromatic or heteroaromatic bonds;

R_x is a reactive group;

S_x is a conjugated substance;

R^6 through R^9 and R^{16} through R^{19} are independently H, $-L-R_x$; or $-L-S_x$; or amino, sulfo, trifluoromethyl, or halogen; or C_1-C_6 alkyl, C_1-C_6 alkoxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino,

- or hydroxy; or any two adjacent substituents of R⁸ through R⁹ and R¹⁸ through R¹⁹ form a fused benzo ring that is optionally substituted one or more times by -L-R_x; or by -L-S_z; or by amino, sulfo, trifluoromethyl, or halogen; or by C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ alkylamino, C₂-C₁₀ dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy;

R² and R¹² are independently a C₁-C₆ alkyl or C₁-C₆ alkyl substituted by sulfo;

R¹³ and R¹⁴ are independently a C₁-C₆ alkyl;

10

n is 1, 2 or 3.

18. A compound, as claimed in Claim 17, wherein R¹⁷ is H, sulfo, or Br, and R¹⁶, R¹⁸, and R¹⁹ are H.

15

19. A compound, as claimed in any of Claims 15-18, wherein R⁷ is sulfo, and R⁶, R⁸, and R⁹ are H; or R⁶ and R⁷ combine to form a fused benzo ring that is substituted one or more times by sulfo, and R⁸ and R⁹ are H.

20

20. A compound, as claimed in any of Claims 1-19, wherein, L is a covalent linkage that incorporates the formula -(CH₂)_d(CONH(CH₂)_e)_{z'}, or -(CH₂)_d(CON(CH₂)_eNH(CH₂)_e)_{z'}, -(CH₂)_d(CONH(CH₂)_eNH)_{z'}, -(CH₂)_d(CONH(CH₂)_eNHCO)_{z'}, where d is 0-5, e is 1-5 and z' is 0 or 1;

25

21. A compound, as claimed in any of Claims 1-20, wherein R⁴ is a C₁-C₆ alkyl.

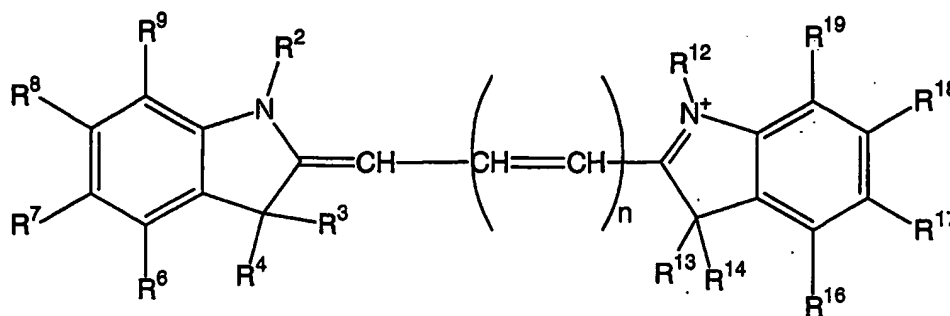
22. A compound, as claimed in any of Claims 1-6 and 8-20, wherein there is only one R_x on the compound and it is an activated ester of a carboxylic acid, an amine, a carboxylic acid, a halotriazine, a hydrazide, a maleimide, or a reactive platinum complex.

30

23. A compound, as claimed in any of Claims 1-22, where -L is -(CH₂)_d(CONH(CH₂)_e)_{z'}.

24. A compound, as claimed in any of Claims 1-23, wherein $-R_x$ is a carboxylic acid, or a succinimidyl ester of a carboxylic acid, or a maleimide.
25. A compound, as claimed in any of Claims 1-4 and 6-21, wherein S_i is a conjugated
5 substance that is an antibody or fragment thereof, a fluorescent protein, a lectin, an oligonucleotide, a small-molecule drug, or a tyramide.
26. A compound, as claimed in any of Claims 1-25, where $n=1$.
- 10 27. A compound, as claimed in any of Claims 1-25, where $n=2$.
28. A compound, as claimed in any of Claims 1-25, where $n=3$.

29. A compound of the formula



5 and its salts, wherein

R² is -L-R_x or -L-S_y; and R⁴, R¹³, and R¹⁴ are methyl;

L is a covalent linkage that is -(CH₂)_e(CONH(CH₂)_z)_f-, e is 1-5 and z is 0 or 1;

10

R_x is a reactive group that is a carboxylic acid, a succinimidyl ester of a carboxylic acid or a maleimide;

S_y is a conjugated substance that is an antibody or fragment thereof, a fluorescent protein, a lectin, a nucleotide, an oligonucleotide, a peptide, a small-molecule drug, or a tyramide;

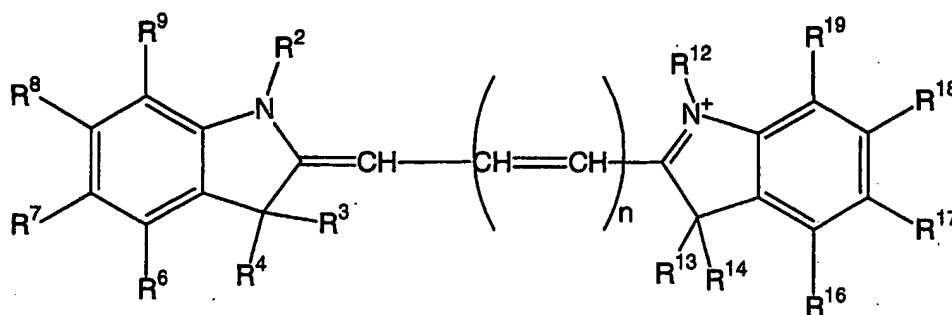
15

R³ and R¹² are independently methyl or sulfopropyl;

20 R⁷ and R¹⁷ are sulfo, and R⁶, R⁸, R⁹, R¹⁶, R¹⁸, and R¹⁹ are H; and

n is 1.

30. A compound of the formula:



5 and its salts, wherein

R³ is -L-R_x or -L-S_y; and R⁴, R¹³, and R¹⁴ are methyl;

10 L is a covalent linkage that is -(CH₂)_e(CONH(CH₂)_{z'})_f, e is 1-5 and z' is 0 or 1;

R_x is a reactive group that is a carboxylic acid, a succinimidyl ester of a carboxylic acid or a maleimide;

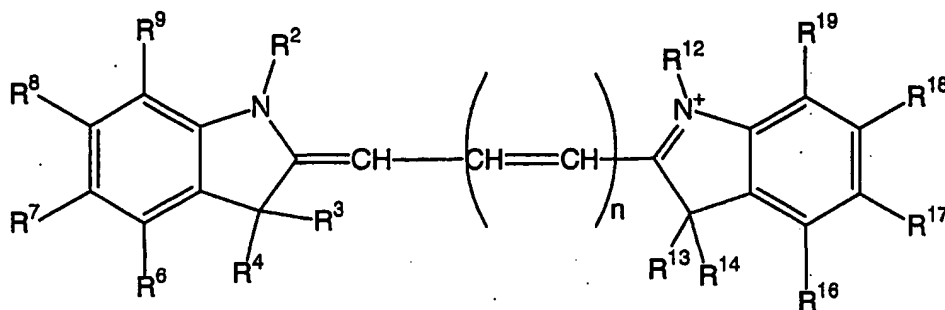
15 S_y is a conjugated substance that is an antibody or fragment thereof, a fluorescent protein, a lectin, a nucleotide, an oligonucleotide, a peptide, a small-molecule drug, or a tyramide;

R² and R¹² are independently methyl or sulfopropyl;

20 R⁷ and R¹⁷ are sulfo, and R⁶, R⁸, R⁹, R¹⁶, R¹⁸, and R¹⁹ are H; and

n is 2.

31. A compound of the formula:



5 and its salts, wherein

R³ is -L-R_x or -L-S_y; and R⁴, R¹³, and R¹⁴ are methyl;

10 L is a covalent linkage that is -(CH₂)_e(CONH(CH₂)_{z'})_z-, e is 1-5 and z' is 0 or 1;

R_x is a reactive group that is a carboxylic acid, a succinimidyl ester of a carboxylic acid or a maleimide;

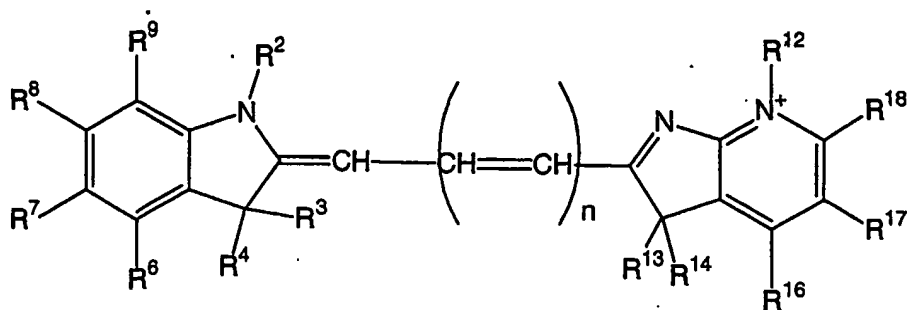
15 S_y is a conjugated substance that is an antibody or fragment thereof, a fluorescent protein, a lectin, a nucleotide, an oligonucleotide, a peptide, a small-molecule drug, or a tyramide;

R³ and R¹³ are independently methyl or sulfopropyl;

20 R⁷ and R¹⁷ are sulfo, and R⁶, R⁸, R⁹, R¹⁶, R¹⁸, and R¹⁹ are H;

n is 3.

32. A compound of the formula:



5 and its salts, wherein

R^9 is $-L-R_x$ or $-L-S_z$; and R^4 , R^{13} and R^{14} are methyl;

10 L is a covalent linkage that is $-(CH_2)_e(CONH(CH_2)_{z'})_e-$, e is 1-5 and z' is 0 or 1;

R_x is a reactive group that is a carboxylic acid, a succinimidyl ester of a carboxylic acid or a maleimide;

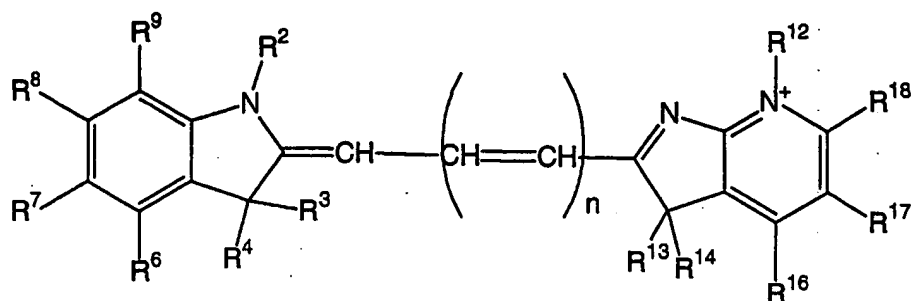
15 S_z is a conjugated substance that is an antibody or fragment thereof, a fluorescent protein, a lectin, a nucleotide, an oligonucleotide, a peptide, a small-molecule drug, or a tyramide;

R^6 , R^8 , R^9 , R^{16} , R^{17} and R^{18} are H; R^7 is sulfo;

20 R^3 and R^{12} are independently methyl or sulfopropyl;

n is 2.

33. A compound of the formula:



and its salts, wherein

5

R^8 is $-L-R_x$ or $-L-S_z$; and R^4 , R^{18} and R^{14} are methyl;

L is a covalent linkage that is $-(CH_2)_e(CONH(CH_2)_{z'})_e$, e is 1-5 and z' is 0 or 1;

10

R_x is a reactive group that is a carboxylic acid, a succinimidyl ester of a carboxylic acid or a maleimide;

S_z is a conjugated substance that is an antibody or fragment thereof, a fluorescent protein, a lectin, a nucleotide, an oligonucleotide, a peptide, a small-molecule drug, or a tyramide;

15

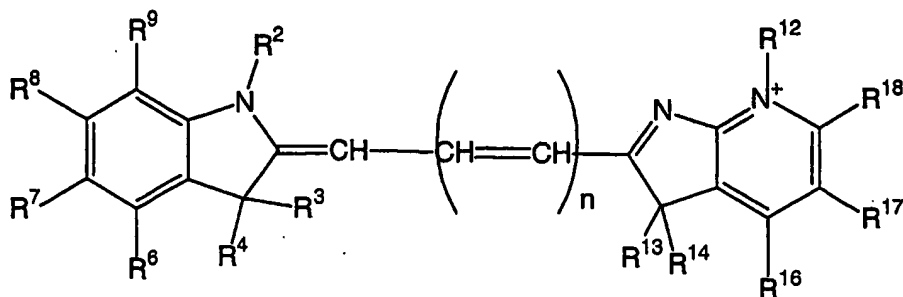
R^6 , R^8 , R^9 , R^{18} , and R^{16} are H; R^7 is sulfo; R^{17} is Br;

R^2 and R^{12} are independently methyl or sulfopropyl;

20

n is 2.

34. A compound of the formula:



and its salts, wherein

5

R^3 is $-L-R_x$ or $-L-S_z$; and R^4 , R^{13} and R^{14} are methyl;

L is a covalent linkage that is $-(CH_2)_e(CONH(CH_2)_{z'})_f-$, e is 1-5 and z' is 0 or 1;

10

R_x is a reactive group that is a carboxylic acid, a succinimidyl ester of a carboxylic acid or a maleimide;

S_z is a conjugated substance that is an antibody or fragment thereof, a fluorescent protein, a lectin, a nucleotide, an oligonucleotide, a peptide, a small-molecule drug, or a tyramide;

15

R^9 and R^7 combine to form a fused benzo ring, which benzo ring is substituted meta and para to R^7 attachment site by sulfo; R^8 , R^9 , R^{13} , and R^{14} are H; R^{17} is Br;

20 R^2 and R^{13} are independently methyl or sulfopropyl;

n is 2.

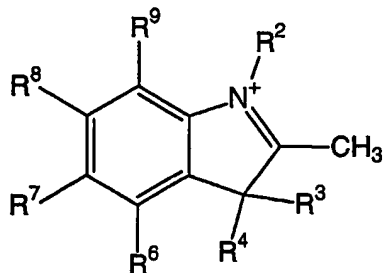
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35. A method of staining a biological sample, comprising:

combining a dye solution comprising any of the dyes described in Claims 1-34, with a biological sample in a concentration sufficient to yield a detectable optical response under the desired conditions.

36. A method, as claimed in Claim 43, wherein the sample comprises cells.
37. A method, as claimed in Claim 43, wherein the sample comprises proteins or
 5 nucleic acid polymers in a microarray.
38. A method, as claimed in Claim 43, wherein the sample comprises a ligand that is a member of a specific binding pair, for which S_i is a complementary member.
- 10 39. A method, as claimed in any of Claims 35-38, wherein the sample is contained in or on a solid or semi-solid matrix that is a membrane, an electrophoretic gel, a silicon chip, a glass slide, a microwell plate, or a microfluidic chip.
40. A kit for fluorescent labeling of a biological sample, comprising
 15 a dye solution comprising any of the compound described in Claims 1-34, or its salts; and
 a buffer suitable for use with such sample.

41. A compound of the formula:



and its salts, wherein

- 25 R^9 is C_3 - C_7 carboxyalkyl; and R^4 is a C_1 - C_6 alkyl;
- R^2 is H or a C_1 - C_6 alkyl or C_1 - C_6 alkyl substituted by sulfo.

- R⁶ through R⁹ are independently H, amino, sulfo, trifluoromethyl, or halogen; or C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy; or any two adjacent substituents of R⁶ through R⁹ form a fused benzo ring that is optionally substituted one or more times by amino, sulfo, trifluoromethyl, or halogen; or by C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino, each of which is optionally further substituted by carboxy, sulfo, amino, or hydroxy.

42. A compound, as claimed in Claim 41, wherein
- 10 R³ is C₆-C₆ carboxyalkyl; and R⁴ is methyl;

R² is methyl or sulfopropyl;

- R⁶ and R⁷ form a fused benzo ring that is optionally substituted one or more times by sulfo; or R⁷ is independently H or sulfo, and R⁶ is H; both R⁸ and R⁹ are H.
- 15

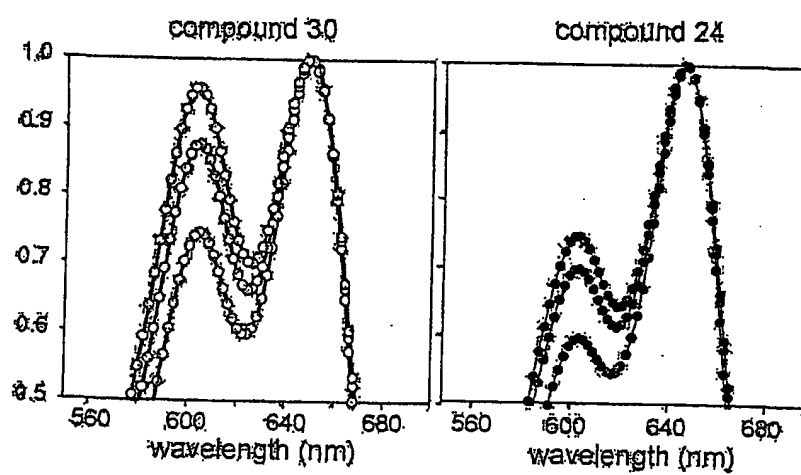


Figure 1

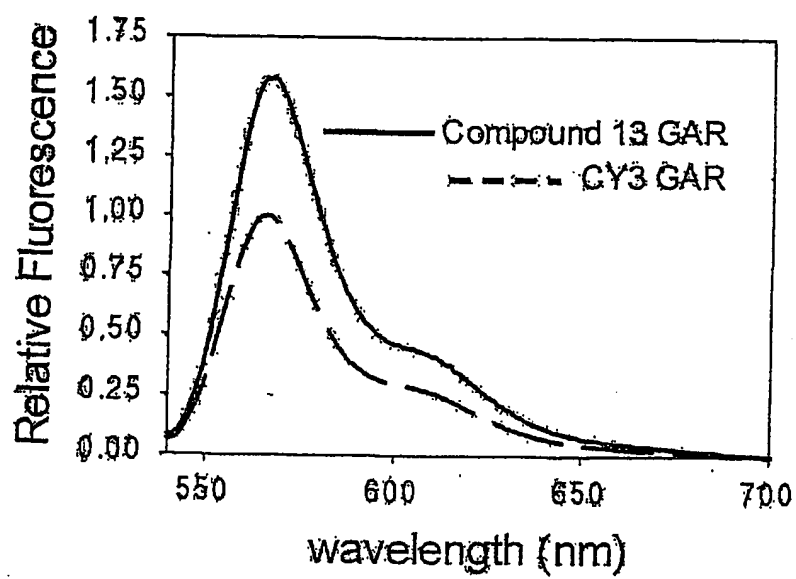


Figure 2

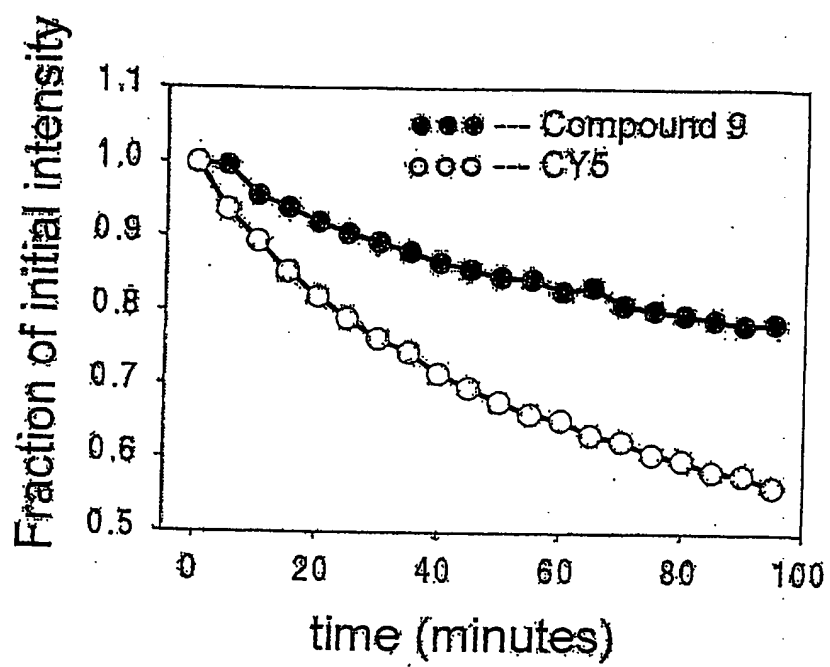


Figure 3

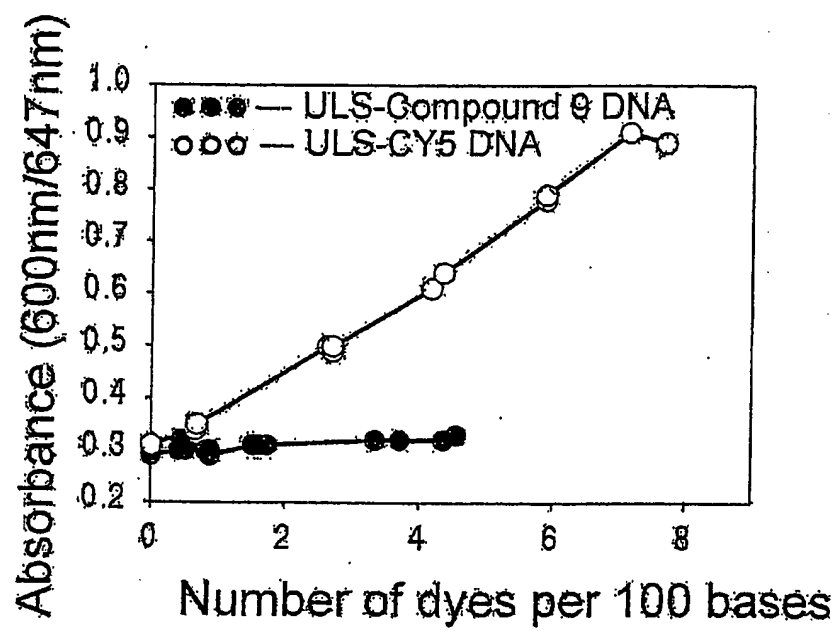


Figure 4

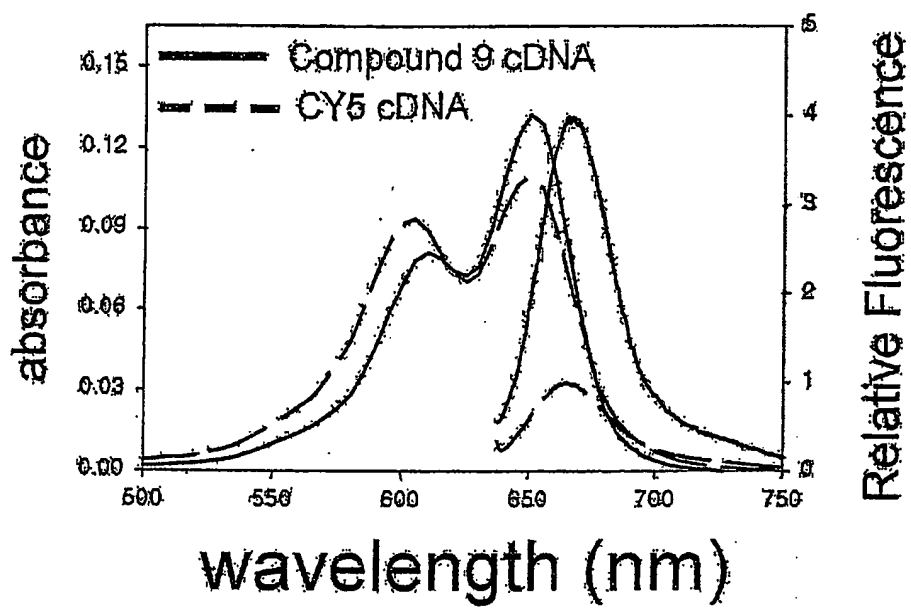


Figure 5